CHAPTER III

MATERIALS AND METHODS

1. Plant Material

Samples of senna pods were kindly supplied by the Government Pharmaceutical Organization of Thailand and the Thai Commodity Co.Ltd, Bangkok. All of them were sun-dried and collected mainly from the plantation sites in Amphor Pattananikom, Lop-Buri province and some were from Amphor Chan tuek, Nakhon Ratchasima province. The *Cassia angustifolia* Vahl seeds used for callus initiation were kindly provided by Mr. Inson Klongkan-ngan. The seeds were collected from the plants in Lop-Buri province and germinated in the Tissue Culture Laboratory, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

2. Chemicals and Apparatus

Reference compounds of sennoside A and B were purchased from Extrasynthese (Z.I. Lyon Nord, Genay-France). Standard chrysarobin which is composed of chrysophanol and physcion was a gift from Faculty of Pharmacy, Mahidol University. Purification of chrysophanol

and physcion from chrysarobin was carried out by using column chromatography and preparative TLC (Silica gel, solvent system : petroleum ether : ethyl acetate (90:5). The band of chrysophanol and physcion were eluted and recrystallized to obtain pure crystals of the two compounds. Various plant hormones and premixed MS and B5 media were purchased from Gibco Laboratories. All solvents were of analytical grade and were obtained from Merck (Damstadt, Germany). Silica gel 60 F 254 pre-coated plates and silica gels 60 (number 9385) particle size 0.040-0.063 mm (230-400 mesh ASTM) for column chromatography were purchased from Merck (Damstadt, Germany). The solid-phase extraction was performed on an Adsorbex SPU chamber, Merck (Damstadt, Germany) connected with vacuum pumping system. The UV-VIS spectrophotometer model Lambda 3b was obtained from Perkin-Elmer (U.S.A.). The Shimadzu dual wavelength thin layer chromato scanner model CS-930 obtained from Kyoto, Japan.

3. Development of Solid Phase Extraction Spectrophotometry for Rapid Screening of High Sennoside-Producing Plants

3.1 Extraction of Sennosides from *C. angustifolia* Pods

Samples of dried senna pods were powdered by using IKA-10 blender (Janke & Kunned GmbH & Co.KG, Germany) and passed through a seive no. 170 before use. Fifty mg amount of each powder sample was then extracted

under reflux with 10 ml methanol for 30 min in a 20 x 2.5 cm tube connected with a 15 cm condenser. After cooling and precipitating of the extracted powder one milliliter of the clear extract was taken for sample cleaning as described in the following section.

3.2 Sample Cleaning of Senna Pod Extracts by Solid-Phase Extraction

One milliliter of the clear extract obtained from above was mixed with 1 ml ethyl acetate. The mixture was then loaded onto a minicolumn of 600 mg silica gel which had been pre-equilibrated with 5 ml of methanol : ethyl acetate (1:1).The column was washed with 12 ml of the same solvent to get rid of impurities before eluted with 9 ml of methanol to obtain sennoside fraction.

3.3 Compositional Analysis of Sennoside Fraction by HPLC

The sennoside fraction obtained after solid-phase extraction was analysed for it chemical composition by HPLC. The HPLC apparatus consist of a Varian Trister Model 9010 solvent delivery system attached to a model 9050 variable wavelength detector and model 4400 integrator (varian, USA).Separation were achieved by using a reversed phase C_{18} stainless-steel column (Varian SP-C_{18}, 15 cm x 4 mm I.D., particle size 5 μm). The analysis was carried out at room temperature using a gradient elution from a 10%. Solution of acetonitrile

in water to a mixture of 30% methanol in water for 30 minutes. Injection volumn of each standard solution or sample was 20 μl. Detection was at 360 nm with the flow rate of 1 ml/min.

3.4 Determination of Total Sennosides by Spectrophotometric Method

The sennoside fraction obtained after solid-phase extraction was determined for its total sennosides by spectrophotometric method using the wavelength of 360 nm and the calibration curve of sennoside B. In constructing the standard curve, the solution of sennoside B was prepared in methanol to obtain the concentration of 1.0 mg/ml (protected from light). This stock solution was half diluted in a stepwise manner to obtain the concentration range of 0.35-10 μg/ml. Each sennoside B solution was read for its absorbance at 360 nm.

3.5 Determination of Total Sennosides by British Pharmacopoeia Method

About 0.15 g, in No 180 powder of senna pods is weighed accurately into 100 ml. round bottom flask. After addition of 30.0 ml. water and shaking, the flask is weighed and subsequently heated in water bath for 15 min with refluxing. After cooling, the flask is weighed again and the weight is brought to the initial value by adding water. This is follow by centrifuging of the liquid transfer 20.0 ml of supernatant liquid to a 150 ml

separating funnel add 0.1 ml of 2 M hydrochloric acid, shake with 15 ml quanties of chloroform three times. After separation of the two layer the chloroform phase is discarded. The aqueous phase is added 0.1 g of sodium hydrogen carbonate and shaked for 3 minutes and centrifuged after that 10.0 ml of the supernatant liquid is transfered into a 100 ml round bottomed flask fitted with ground-glass neck. Add 20 ml of a 10.5% w/v solution of iron (III) chloride hexahydrate, mix, heat under a reflux condenser in a water-bath for 20 minutes, add 1 ml of hydrochloric acid and continue heating for 20 minutes, shaking frequently, until the precipitate is dissolved. After cooling the mixture is transfered to a separatory funnel and extract three times with 25 ml portion of ether which was used to prerinse the flask. The three ether extracts are combined and washed twice with 15 ml portion of water. The ether extracts are now transfered into a 100 ml volumetric flask and brought to 100.0 ml with ether. Of this solution 10.0 ml is evaporated to dryness (round-bottom flask in water jet vaccum) dissolve the residue into 10 ml by 10 ml of a 0.5% w/v solution of magnesium acetate in methanol. Measure the absorbance of the resulting solution at 515 nm. using methanol in the reference cell. Calculate the percentage content of hydroxyanthracene glycosides express as sennoside B taking 240 as the value of A (1%, 1 cm) at the maximum at 515 nm. The British Pharmacopoeia Method can be explained with time course in flow charge (below) this can be said that

at least 4 hours must be spend for one experiment.

Table 7. Analysis of Sennoside by British Pharmacopoeia Method and time course

time course (min)	Procedure
5	180 mesh powder with 30 ml of water (weigh)
	↓
15	heat under reflux condenser in water bath 15 min
	↓
10	allow to cool weigh and restore to the original weigh with water
	↓
10	centrifuge
	↓
5	transfer 20.0 ml supernatant to 150 ml funnel
	↓
30	add 0.1 ml of 2 M HCl acid, shake with 15 ml $CHCl_3$ 3 times (allow layers to separate discard $CHCl_3$ layer)
	↓
5	add 0.1 g of Sodium hydrogen carbonate shake 3 min
	↓
10	centrifuge
	↓

Table 7. (Continued)

time course (min)	Procedure
	↓
3	transfer 10 ml supernatant to 100 ml round-bottomed flask
	↓
2	add 20 ml of 10.5% w/v Solution of Iron (III) Chloride hexahydrate
	↓
20	mix, heat under reflux condenser in water bath for 20 min
	↓
30	Add 1 ml HCl acid. Continue heating 20 Min
	↓
20	cool transfer mixture to separating funnel
	↓
30	shake with three 25 ml quantify of ether
	↓
20	combine ether extract wash with water 15 ml 2 times
	↓
20	dilute extract of ether to 100 ml
	↓
20	evaporate 10 ml to dryness
	↓

Table 7. (Continued)

time course (min)	Procedure
5	↓ dissolve the residue into 10 ml with 10 ml of a 0.5% w/v solution of magnesium acetate in methanol ↓
10	measure absorbance mase 515 nm
total 260	

4. Plant Tissue Culture Techniques

4.1 Preparation of *Cassia angustifolia* Leaf Explants

Callus cultures of *Cassia angustifolia* were initiated by using young leaves of the plant as starting materia. Before initiating the callus, the surface of the explants were sterilized as describe below.

young leave of *Cassia angustifolia*

↓

cleaned with tap water

↓

dipped into 15% Clorox® solution

and Shaked gently for 15 min

↓

washed with distilled water 2 times in laminar flow cabinet

↓

cut into small pieces with a sharp scalpel

↓

transfered to solid nutrient medium

(aseptic technique)

4.2 Medium Preparation

The culture media of B5 and MS, as shown in Table 8, were prepared from commercially available premixed media. Various stock solutions of hormones used for B5 and MS were prepared at concentrations shown in Table 9. The culture media were then prepared by mixing the stock solution and add 3% sucrose as described in table 10. The pH of each medium was adjusted to its desired value with 1N potassium hydroxide or 1N hydrochloric acid. The media were heated on water bath and 0.8% w/v agar added to make solid media. This step was not needed for preparing liquid media. The media were sterilized by autoclaving using the conditions of 121°C,

15 lb/in^2 for 15 minutes. The commercially prepared media were as follows.

4.2.1. Preparation of B5 medium from Gibco® prepared medium

4.2.1.1 Dust the powder into distilled water about 500 ml and stir continuously.

4.2.1.2 Rinse out inside of container to remove all trace of powder by distilled water.

4.2.1.3 For liquid medium, adjust pH if necessary to 5.0 and for solid medium check pH if necessary to 5.7, then add agar and heat until the agar solute in medium completely after adjusting the volume.

4.2.1.4 Dilute to final volume of 1 liter.

4.2.1.5 For solid medium, heat gentlely with continuous mixing until the solution clears.

4.2.1.6 Dispense desired amount of medium into culture vessels, for liquid medium dispense 50 ml of medium into 250 ml Ertenmyer flask, for

solid medium dispense the medium into the bottle which have space suitable to use the quantity of medium are 10 ml approximately.

4.2.1.7 autoclave for 15 minute at 15 lb/in^2, 121°C.

4.2.2. Preperation of MS medium from Gibco prepared medium

4.2.2.1 Dust the powder into distilled water about 500 ml and stir continuously.

4.2.2.2 Rinse out inside of container to remove all trace of powder by distilled water.

4.2.2.3 The content of this package have been adjusted to pH 5.7 $\pm$ 0.1 No further adjustment necessary.

4.2.2.4 Dilute to final volume of 1 liter.

4.2.2.5 For solid medium, heatgentlely with continuous mixing until the solution clears.

4.2.2.6 Dispense desire amount of medium into culture vessels.

4.2.2.7 Autoclave for 15 minute at 15 lb/in^2, 121°C

Table 8. Inorganic Salt and Vitamin Compositions of Plant Tissue Culture Media

Constituent	Concentration (mg/liter)	
	MS	B5
Macronutrients :		
$MgSO_4.7H_2O$	370	250
KH_2PO_4	170	-
$NaH_2PO_4.H_2O$	-	150
KNO_3	1900	2500
NH_4NO_3	1650	-
$CaCl_2.2H_2O$	440	150
$(NH_4)_2.SO_4$	-	134
Micronutrients :		
H_3BO_3	6.2	3
$MnSO_4.H_2O$	15.6	10
$ZnSO_4.2H_2O$	8.6	2
$NaMoO_4.2H_2O$	0.25	0.25
$CuSo_4.5H_2O$	0.025	0.025
$CoCl_2.6H_2O$	0.025	0.025
KI	0.83	0.75
$FeSO_4.7H_2O$	27.8	-
Na_2EDTA	37.3	-
EDTA Na Ferric	-	40

Table 8. (Continued)

Constituent	Concentration (mg/liter)	
	MS	B5
Sucrose (g)		30
Vitamins :		
Thiamine HCl	0.5	20
Pyridoxine HCl	0.5	1
Nicotinic acid	0.5	1
myo-Inositol	100	100
pH	5.8	5.5

Table 9. Preparation of Stock Solution of B5 and MS Media

B5		MS		Remarks
Stock 1 (Macronutrients)	g/1000 ml	**Stock 1 (Macronutrients)**	g/1000 ml	store in refrigerator
$NaH_2PO_4 \cdot H_2O$	3.00	NH_4NO_3	33.0	
$(NH_4)_2SO_4$	2.68	KNO_3	38.0	
$MgSO_4 \cdot 7H_2O$	5.00	$MgSO_4 \cdot 7H_2O$	7.4	
KNO_3	50.0	KH_2PO_4	3.4	
Stock 2 (Micronutrients)	mg/100 ml	**Stock 2 (Micronutrients)**	mg/100 ml	store in refrigerator
$MnSO_4 \cdot H_2O$	1000	H_3BO_3	620	
H_3BO_3	300	$MnSO_4 \cdot H_2O$	1690	
$ZnSO_4 \cdot 7H_2O$	200	$ZnSO_4 \cdot 7H_2O$	860	
$Na_2MoO_4 \cdot 2H_2O$	25	$Na_2MoO_4 \cdot 2H_2O$	25	
$CuSO_4 \cdot 5H_2O$	2.5	$CuSO_4 \cdot 5H_2O$	2.5	
$CoCl_2 \cdot 6H_2O$	2.5	$CoCl_2 \cdot 6H_2O$	2.5	
Stock 3 (Ca Stock)	g/100 ml	**Stock 3**	g/100 ml	store in refrigerator
$CaCl_2 \cdot 2H_2O$	3.0	$CaCl_2 \cdot 2H_2O$	8.7	
Stock 4 (KI stock)	mg/100 ml	**Stock 4** the same as for B5		store in amber bottle in refrigerator
KI	75			
Stock 5 (Vitamins)	mg/100 ml	**Stock 5 (Vitamins)**	mg/100 ml	store in freezer (25-ml fraction)
Nicotinic acid	100	Thiamine. HCl	8	
Thiamine. HCl	1000	i-Inositol	10,000	
Pyridoxine. HCl	100			
i-Inositol	10,000			
Stock 6 (Fe-EDTA stock)	g/500 ml	**Stock 6** the same as for B5		store in refrigerator
Na_2EDTA	3.73			
$FeSO_4 \cdot 7H_2O$	2.78			
2,4-D stock solution (100 mg/l)	mg/100 ml	**2,4-D stock solution** the same as for B5		dissolve 2,4-D in 5 ml ethanol ; heat slightly and gradually dilute to 100 ml with water
2,4-D	10			
NAA stock solution (100 mg/l)		**NAA stock solution** the same as for B5		
NAA	10			
Kinetin stock solution (100 mg/l)		**Kinetin stock solution** the same as for B5		dissolve kinetin in a small volume of 0.5 N HCl by heating slightly and gradually dilute to 100 ml with distilled water. Store in refrigerator.
Kinetin	10			

Table 10. Preparation of B5 and MS Media

Condition	B5	MS
Distilled Water	1000 ml	1000 ml
Stock 1	50 ml	50 ml
Stock 2	1.0 ml	1.0 ml
Stock 3	1.0 ml	2.9 ml
Stock 4	1.0 ml	1.0 ml
Stock 5	1.0 ml	1.0 ml
Stock 6	5.0 ml	5.0 ml
Sucrose	30 g	30 g
Agar (solid medium)	8 g	8 g
Auxin (100 mg/l)	as needed	as needed
Cytokinin (100 mg/l)	as needed	as needed
Final pH adjust to	pH 5.5	pH 5.8

4.3 Study on the Effect of Hormonal Factors on Callus Formation

Various types and concentrations of hormones in B5 and MS media were varied to study their effects on callus formation and plant regeneration from *Cassia angustifolia* leaf explants. Two cytokinins (Kinetin and BA) at the concentration of 1.0 mg/l combined with three auxins (NAA; 2,4-D and IAA) at the concentrations between 0.1 and 1.0 mg/l were used with 3% sucrose (w/v) and 0.8% w/v agar. The callus formation and plant regeneration in each medium were observed periodically and the results were recorded. The combination of hormone was shown in table 8.

Table 11. The combination of Auxins and Cytokinins in various tested Culture Media.

Concentration of Cytokinins	Concentration of Auxins (mg/l)		
	NAA	2,4-D	IAA
BA 1.0 mg/l	0.1	-	-
	0.5	-	-
	1.0	-	-
	-	0.1	-
	-	0.5	-
	-	1.0	-

Table 11. (Continued)

Concentration of Cytokinins	Concentration of Auxins (mg/l)		
	NAA	2,4-D	IAA
Kinetin 1.0 mg/l	-	-	0.1
	-	-	0.5
	-	-	1.0
	0.1	-	-
	0.5	-	-
	1.0	-	-
	-	0.1	-
	-	0.5	-
	-	1.0	-
	-	-	0.1
	-	-	0.5
	-	-	1.0

4.4 Establishment of Suspension Cultures

Suspension cultures of *C. angustifolia* were initiated by using callus cultures maintained in B5 medium supplemented with 3% (w/v) sucrose, 1 mg/l BA and 0.5 mg/l 2,4-D. The quantity of the medium was 50 ml in a 250 ml Erlenmeyer flask. The flasks containing suspension were incubated on a rotary shaker rotated continuously at 120 r.p.m. at 25°C. After obtaining stable *C.*

angustifolia cell cultures, the cell suspensions were maintained under the same conditions and subcultured every 2 weeks, in the same medium.

4.5 Study on Culture of Growth *C. angustifolia* Cell Cultures

A large number of flasks of *C. angustifolia* suspension cultures were prepared for studying the growth and anthraquinone production of the cultures the procedure was collecting the cell from suspension culture by filtration and washing the cell by distilled water and drying in hot air oven under temperature 50°C until it dried. In the same samples the amount of anthraquinone was examined.

5. Detection and Determination of Anthraquinones in *C. angustifolia* Suspension Culture

5.1 Extraction of Anthraquinones from *C. angustifolia* Suspension Culture

Four hundred milligrams fresh weight of the suspension culture were inoculated in liquid B5 medium supplement with BA 1.0 mg/l, 2,4-D 0.5 mg/l and 3% w/v sucrose. The suspension culture were harvested within 40 days by suction filtration. The tissues were dried in a hot air oven with controlled temperature at 50°C for two days.

Each sample of dry tissue was *C. angustifolia*

(Ca. 50 mg) was ground and extracted under reflux with 50 ml petroleum ether for 1 hour. After filtering the filtrate was adjusted to 50 ml. Four milliliters of the crude extract were used for qualitative and quantitative analysis of anthraquinones.

5.2 Sample Cleaning of *Cassia angustifolia* Cell Culture Extract by Solid-Phase Extraction

Four millilites of the crude extracts obtained as described above was evaporated until dryness. The dried extract was then dissolved in 1 ml petroleum ether : ethyl acetate, ratio 9:1. To separate anthraquinones from other compounds, minicolumns of filled with silica gel (600 mg) were used. The column were first pre-equilibrated with petroleum ether and ethyl acetate ratio 9:1. One milliliter of the prepared samples was then transfered into the column, followed by washing with 4 ml of the same solvent. Anthraquinones which are not bound to the column are passed through the minicolumn quantitatively within this 4 ml. This anthraquinone fraction was estimated for total anthraquinone content by spectrophotometric method (section 5.4.3).

5.3 Detection of Anthraquinones in *C. angustifolia* Cell Suspension Cultures

The ethanolic extract of *C. angustifolia* cultures prepared as described in the section 5.1 was tested for the presence of various chemical groups. The

screening concluded alkaloid test, (Dragendorff's reagent, Mayer's reagent, Valser's reagent, Wagner's reagent, Marme's reagent, Hayer's reagent), cardiac glycoside test, (Kedde's test, Liebermann-Burchard test, Keller-Kiliani test), cyanogenetic test (sodium picrate paper test), tannin test (gelatin solution, ferric chloride, bromine water), flavonoid test (cyanidin test, leucoanthocyanin) and anthraquinone test (Modified Borntrager test). Since the only anthraquinone test was found to be positive, further identification of the anthraquinones was carried out. This was done by using the technique of TLC and Mass spectroscopy.

5.3.1. TLC Conditions

Technique	: one way, ascending, double developement
Absorbent	: aluminium sheet silica gel 60 F254 (Precoated, Merck)
Plate size	: 10 x 5 cm.
Layer thickness	: 0.2 mm.
Solvent system	: Pet ether : Ethyl acetate 9:1
Sample size	: 5 μl
Distance	: 7 cm
Temperature	: 25 - 30°C
Detection	: Ultraviolet light at 254 nm. (short wave) 365 nm. (long wave)
Standards used	: Emodin, Alizarin, Chrysophanol, Physcion, Sennoside B

The identity of chrysophanol and physcion on the TLC was also confirmed by other systems of TLC using various solvent such as chloroform ether, pet ether, Ethyl acetate, methanol and hexane in various ratio.

5.3.2. Mass spectroscopy

Column chromatography and preparative TLC was used in order to obtain pure chrysophanol and physcion. The fractions from column chromatography was continued purifying by preparative TLC using same solvent system as TLC (Petroleum ether : Ethyl acetate (9:1). The compounds could be purified to the amount that was sufficient for mass spectral analysis by crystallization the fractions which expect to be chrysophanol and physcion. The crystals of expected chrysophanol and expected physcion were identified by mass spectroscopy.

5.4 Quantitative Analysis of Anthraquinones (Chrysarobin and Physcion)

The solution of pure chrysophanol solution with the concentration of 10 μg/ml was prepared in petroleum ether. From this stock solution, various concentrations of chrysophanol were prepared by half dilution and a standard curve in the range of 0.1-6 μg/ml was then constructed. The absorbance was measured at 290 nm, using petroleum ether as reference. The anthraquinone content in the cleaned extracts of *C. angustifolia* culture using the standard curve of chrysophanol.