CHAPTER III

MATERIALS AND METHODS

1. Chemicals

Twenty-three authentic isoquinoline alkaloids, including (-)-asimilobine, (-)-asimilobine-2-*O*-β-*D*-glucoside, (-)-anonaine, (-)-isolaureline, (-)-roememeroline, (-)-dicentrine, (-)-nordicentrine, (-)-phanostenine, cassythicine, magnoflorine, (-)-xylopine (-)-tetrahydropalmatine, (-)-capaurine, (-)-thaicanine, (-)-corydalmine, (-)-xylopinine (-)-N-methyltetrahydropalmatine,(-)-tetrahydrostephabine, (+)-reticuline,(±)-oblongine (-)-delavaine and (-)-salutaridine were isolated from tubers of *Stephania pierrei* by Dr. Kittisak Likhitwitayawuid, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

Organic solvents used in this phytochemical study were all commercial grade or better.TLC plates of silica gel 60 F_{254} 0.2 mm thick on aluminium sheet were purchased from Merck (Damstadt, Germany). The chemicals for culture media were all tissue culture grade. Various plant growth regulators were obtained from Gibco Laboratories (New York, USA) and gelling agents (agar and gellan gum) were purchased from Difco Laboratories (Detroit Michigan, USA) and Sigma Chemical Co. (St.Louis, Mo, USA). Water was triple deionized and distilled in glass.

2. Plant tissue culture technique

2.1 Plant material

Tubers of *Stephania pierrei* Diels used for this tissue culture work were obtained from Jartujark market, Bangkok in September 1993. The origin of the tubers was said to come from Prachin Buri Province. Authentication was compared with herbarium specimens in the Botany Section, Technical Division, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand

2.2 Nutrient media

Standard basal media used in this study were White media (WT) (White, 1963) and Root media (RT). The composition of these media is shown in Table 4. For plant growth regulators, the following auxins ,cytokinins and gibberellin were used :

auxins : indole-3-acetic acid (IAA), indole-3-butyric acid (IBA), α-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D),

cytokinin : kinetin-6-furfurylaminopurin (kinetin), 6-benzylaminopurine or N^6-benzyladenine (BA) and

gibberellin (GA3).

Table 4 Inorganic salt and vitamin composition of plant tissue culture media

Constituent	Concentration (mg/litre)	
	WT	RT
Macronutrients :		
KCl	65	-
$MgSO_4.7H_2O$	720	370
$NaH_2PO_4.H_2O$	16.5	-
Na_2SO_4	200	-
KNO_3	80	1,900
$Ca(NO_3)_2.4H_2O$	300	-
NH_4NO_3	-	1,650
$CaCl_2.2H_2O$	-	440
KH_2PO_4	-	170
Micronutrients :		
$MnSO_4.4H_2O$	7	22.3
KI	0.75	8.3
$ZnSO_4.7H_2O$	3	8.6
H_3BO_3	1.5	6.2
$Na_2MoO_4.2H_2O$	-	0.25
$CuSO_4.5H_2O$	-	0.025
CoCl2.6H2O	-	0.025

Table 4 (continue)

Constituent	Concentration (mg/litre)	
	WT	RT
Micronutrients (continuous)		
$FeSO_4.7H_2O$	-	27.8
$Fe_2(SO_4)_3$	2.5	-
$NaEDTA.2H_2O$	-	37.3
Vitamins :		
Glycine	3	-
Cysteine	1	-
Thiamine HCl	0.1	0.1
Pyridoxine HCl	0.1	0.5
Nicotinic acid	0.5	0.5
Ca-d-pantothenic acid	1	-
Sucrose (g)	30	30
Agar (g)	8	-
Gellan gum (g)	-	3
Myo-inositol (mg)	-	100
pH	5.6	6

2.3 Media preparation

Various stock solutions of WT, RT and plant growth regulators were prepared at the concentration shown in Table 5.

Table 5 Preparation of stock solution of WT, RT and plant growth regulators

WT		RT	
Stock 1A (Macronutrients)	g/1000 ml	Stock 1 (Macronutrients)	g/1000 ml
KCl	13	NH_4NO_3	33
$MgSO_4.7H_2O$	144	KNO_3	38
$NaH_2PO_4.H_2O$	3.3	$CaCl_2.2H_2O$	8.8
Na_2SO_4	40	$MgSO_4.7H_2O$	7.4
		KH_2PO_4	3.4
Stock 1B (Macronutrients)	g/1000 ml		
KNO_3	16		
$Ca(NO_3)_2.4H_2O$	60		
Stock 2 (Micronutrients)	mg/100 ml	Stock 2 (Micronutrients)	mg/100 ml
$MnSO_4.4H_2O$	280	$MnSO_4.4H_2O$	2,230
KI	30	KI	83
$ZnSO_4.7H_2O$	120	$ZnSO_4.7H_2O$	860
H_3BO_3	60	H_3BO_3	620
		$Na_2MoO_4.2H_2O$	25
		$CuSO_4.5H_2O$	2.5
		$CoCl_2.6H_2O$	2.5
Stock 3 (Fe Stock)	mg/100 ml	Stock 3 (Fe Stock)	mg/100 ml
$Fe_2(SO_4)_3$	100	$FeSO_4.7H_2O$	556
		NaEDTA.$2H_2O$	746
Stock 4 (Vitamins)	mg/100 ml	Stock 4 (Vitamins)	mg/100 ml
Glycine	60	Nicotinic acid	50
Cysteine	10	Thiamine HCl	10
Thiamine HCl	2	Pyridoxine HCl	50
Pyridoxine HCl	2		
Nicotinic acid	10		
Ca-d-pantothenic acid	20		

Table 6 Preparation of WT and RT media

WT		RT	
Distilled water	1,000 ml	Distilled water	1,000 ml
Stock 1A	5 ml	Stock 1	50 ml
Stock 1B	5 ml	Stock 2	1 ml
Stock 2	5 ml	Stock 3	5 ml
Stock 3	5 ml	Stock 4	1 ml
Stock 4	10 ml	Myoinositol	100 mg
Sucrose	30 gm	Sucrose	30 gm
Auxin (100 mg/l)	as needed	Auxin (100 mg/l)	as needed
Cytokinin (100 mg/l)	as needed	Cytokinin (100 mg/l)	as needed
Gibberellin (100 mg/l)	as needed	Gibberellin (100 mg/l)	as needed
Final pH adjust	5.6	Final pH adjust	6.0

The culture media were prepared by mixing the stock solutions and sucrose and myo-inositol were added into the distilled water, as described in Table 6. The pH of each medium was adjusted to its desired value with 0.1 N sodium hydroxide or 0.1 N hydrochloric acid. The media were solidified with 0.8% (w/v) agar or 0.3%(w/v) gellan gum for solid media. The media were sterilized by autoclaving at 121°c (15 lb/in^2) for 15-20 minutes.

2.4 Preparation of *S. pierrei* explants

The tubers of *S. pierrei* were used as explants or starting material for plant tissue culture studies. Before initiating the callus, the surface of the explants was sterilized as follow :

Tuber of *S. pierrei* (about 1 Kg. weight)

↓

cleaned with running tap water

↓

peeled and discarded the top and the bottom

Figure 11 Explant preparation before culturing

cut the middle for the segments approximately 1.0 cm in thickness

↓

dipped the segments into 15% Clorox^{R} solution for 15 minutes

↓

washed with sterile distilled water 2 times

↓

excised the cambial zone in the cubic shape

↓

transfered to solid nutrient medium

2.5 Establishment of callus cultures

2.5.1 Callus induction

The study of callus induction of *S.pierrei* was carried out using three methods. The first was a study on the type of basal medium required for callus formation. The second was a study on the effect of plant growth regulators on callus induction and the third was a study on culture conditions.

2.5.1.1 Effect of basal media

Various basal media including Murashige and Skoog (MS) (Murashige and Skoog, 1962), Gamborg B-5 (B5) (Gamborg *et al.*, 1970), Linsmaier and Skoog (LS) (Linsmaier and Skoog, 1965), Nisch and Nitsch (NN) (Nisch and Nitsch, 1969), White (WT) (White, 1963) and Woody Plant (WPM) (Lloyd and McCown, 1980) were used for this study. All media were supplemented with 30 g/l sucrose, 1.0 mg/l 2,4-D, 0.1 mg/l kinetin and 0.8% (w/v) agar. The tuber explants of *S. pierrei* (1x1x0.5 cm^3) were transfered onto the media and maintained at 25$\pm$2°c under controlled 16-hr photoperiod conditions.

2.5.1.2 Effect of plant growth regulators

White medium containing various combinations of growth regulators were used for callus indication of *S. pierrei* tuber explants. Four auxins (IAA, IBA, NAA

and 2,4-D) combined with two cytokinins (kinetin and BA) were used at the concentrations of 0.1, 0.5, 1.0 and 2.0 mg/l. The callus formation in each medium was observed periodically and the results were recorded.

2.5.1.3 Effect of culture conditions

White medium supplemented with 30 g/l sucrose, 1.0 mg/l NAA, 0.1 mg/l kinetine, 0.2 mg/l BA and 1.0 mg/l GA_3 were used. The *in vitro* cultures of *S. pierrei* were maintained in two culture conditions. The first condition was in a 16-hour photoperiod (2,000 lux) and 8-hour dark at 25±2°c. The other was in a dark room at the same temperature.

2.5.2 Subculturing

The calli of *S. pierrei* were subcultured on White medium containing 30 g/l, 1.0 mg/l NAA, 0.1 mg/l kinetin, 0.2 mg/l BA, 1.0 GA_3 and 0.8% (w/v) agar for proliferation every 4 weeks and maintained at 25±2°c in the dark.

2.6 Root regeneration from callus cultures

After callus induction, the calli were transfered onto RT medium containing 30 g/l sucrose, 0.1 mg/l NAA, 0.1 mg/l kinetine, 0.2 mg/l BA, 1 mg/l GA_3 and 0.3% (w/v) gellan gum for root regeneration. The cultures were maintained by subculturing every 4 weeks on this medium in the dark at 25±2°c. The regenerated roots were then harvested and used for phytochemical study.

3. Phytochemical Techniques

3.1 Preparation of crude extracts from various plant parts and tissue cultures of *S. pierrei.*

The tubers, leaves and cultured root of *S. pierrei* were dried over night in a hot air oven (60°C). The samples about 0.2-10 g were then ground and extracted with 20 ml 95% ethanol by shaking for 24 hrs. After filtering, each filtreate was evaporated in *vacuo* and the obtained syrupy residue was partitioned with 10 ml chloroform and water (4:1) for six times. Both chloroform and water fractions were collected after each

partition. The pooled chloroform fraction after being dried using Na_2SO_4 anhydrous, gave a chloroform extract whereas the pooled water fraction after lyophyllization, gave an aqueous extract.

3.2 Identification of Isoquinoline Alkaloids in the sample extracts.

Thin layer chromatographic (TLC) densitometric analysis was used as the method for identification of isoquinoline alkaloids in the crude extracts of whole plant and tissue cultures of *S. pierrei* compared with twenty three standard isoquinolines. Five-microlitre-aliqout of each extract was spotted on a TLC plate and separated under the following TLC conditions. Where needed, the identity of some isoquinolines on the TLC was confirmed by other TLC solvent systems which are also described below.

3.2.1 Thin Layer Chromatographic Conditions for Isoquinoline Separation.

Technique	:	one way, ascending, single development
Stationary phase	:	aluminium sheet silica gel 60 F_{254} (precoated, Merck)
Plate size	:	10 x 20 cm^2
Layer thickness	:	0.2 mm
Solvent system	:	a) ethyl acetate : methanol (5:1) b) toluene : ethyl acetate : diethylamine (7:2:1) c) chloroform : methanol (9:1)
Sample size	:	5 µl
Distance	:	10 cm
Temperature	:	25-30°C
Detection	:	a) ultraviolet light at 254 nm b) Dragendorff's spraying reagent

3.2.2 TLC Densitometric Analysis

Alkaloid spots, obtained after thin layer chromatography, which had the same Rf values as standard isoquinolines, were studied for their ultraviolet -absorption spectra by using TLC densitometer. Theresulted absorption spectra were then compared with those of standard isoquinolines. The TLC densitometric conditions are as follows:

Instrumental model	:	Shimadsu Dual-wavelength TLC-scanner, Model CS-930
Lamp	:	Deuterium (D_2)
Determination mode	:	absorption
Scan width	:	x = 10.0 mm y = 0.2 mm
Sensitivity	:	medium
Slit width	:	1.2 x 1.2 mm^2
Wavelength scanned range	:	200-370 nm

3.3 Purification of Dicentrine for IR, Mass and ^{1}H-nmr Analysis

The chloroform extract from tissue cultures of *S. pierrei* was streaked on a TLC plate using the same TLC conditions as described above. After being developed with the solvent system of ethyl acetate :methanol (5:1), the band with the Rf value of 0.34 was cut, eluted with chloroform and filtered. The filtrate was evaporated to dryness to obtain yellow syrupy mass.

3.4 Spectroscopy

3.4.1 Infrared absorption spectra were performed on a Shimadzu 440 and FT-IR. The materials were examined in potassium bromide disce (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

3.4.2 Mass spectra were determined on a Finigan Incos 50 double focusing spectrometer for EI. Operating at 70 ev. with inlet temperature 40°C - 200°C (Department of Chemistry, Faculty of Sciences, Mahidol University).

3.4.3 Proton nuclear magnetic resonance (^{1}H-nmr) spectra were obtained with JEOL JMN-A 500 spectrometer (500 MHz). Chemical shifts were reported in ppm scale, using deuterochloroform ($CDCl_3$) as operating solvent (The Scientific and Technological Research Equipment Center, Chulalongkorn University).

4. Quantitative Analysis of Dicentrine in tissue cultures and plant parts of *S. pierrei*

Syrupy mass of chloroform extracts of tubers (163 mg), leaves (30 mg) and cultured roots (18 mg) of *S. pierrei* were dissolved in appropiate volumes of chloroform. Five-microlitre-aliqout of each chloroform solution was then spotted on a TLC plate. The stationary phase was silica gel 60 F_{254} plate and mobile phase was ethyl acetate : methanol (5:1). Dicentrine in each sample on the TLC plate was quantitated by the TLC densitometric method at 315 nm and calculated based on the standard curve of dicentrine (peak area-concentration relationship). The conditions for dicentrine separation and densitometric analysis were the same as describedabove. The standard curve was established from the standard dicentrine using the concentration range from 0.05 to 0.5 mg/ml.