CHAPTER III

MATERIALS AND METHODS

1. Chemicals

Authentic plaunotol was obtained from Kelnac capsules which are manufactured by Sankyo Co., Ltd. (Tokyo, Japan). Organic solvents used in this study were all reagent grade or better. TLC plates of silica gel 60 F254 on aluminium sheets were obtained from Merck (Damstadt, Germany). The chemicals for culture media were all tissue culture grade. Various plant growth regulators were purchased 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. Quantitative Analysis of Plaunotol in *C. sublyratus* Leaves

2.1 Plant Material

Samples of *C. sublyratus* leaves were collected from various parts of Thailand including Rayong, Prachuap Khiri Khan, Nakhon Pathom Bangkok and Chachoengsao. The samples from Rayong were obtained from the Suan Samunprai Somdej Phra Thepratanarajasuda, Petroleum Authority of Thailand. The samples from Nakhon Pathom were from the Medicinal Plant Garden, Faculty of Pharmaceutical Sciences, Mahidol University, Amphor Salaya. In Prachuap Khiri Khan, the *C. sublyratus* plants were collected from Ban Singkhorn, Amphor Muang. In Bangkok, the leaves of

C. sublyratus were obtained from the Faculty of Pharmaceutical Sciences, Faculty of Science and the Nursery House of Chulalongkorn University. In Chachoengsao, the leaf samples were obtained from the Botanical Garden at Khao Hin Sorn. For other species of the genus *Croton*, the samples were either collected from various sources or purchased from traditional drug stores as deseribed in Table 5. All samples were dried overnight in a hot air oven at 50°C. Each sample was analyzed in triplicates for plaunotol content.

Table 5 Various sources of the *Croton* plants and the plant parts used in this study.

Croton plant	Source	Plant part used in this study
Croton sublyratus Kurz	Various parts of Thailand	Leaves
C. oblongifolius Roxb.	Faculty of Pharmaceutical Sciences, Chulalongkorn University	Leaves
C. crassifolius Geisel	Chao Krom Per Traditional Drug Store	Dried roots
C. caudatus Geisel	Chao Krom Per Treaditional Drug Store	Dried stems
C. tiglium Linn.	Suan Samunprai Somdej Phra Thepratanarajasuda, Petroleum Authority of Thailand, Rayong	Leaves
C. cascarilloides Raeush	Kanchanaburi	Leaves
Croton sp. (Char plao nam ngoen)	Kanchanaburi	Leaves

2.2 Sample Preparation

The dried leaves of *C. sublyratus* were ground to fine powder in a grinder connected with a cool water circulator (IKA-Labortechnik A10 blender, Janke & Kunkel Gmbh & Co. Kg, Germany). After passing a seive no. 40, two hundred miligrams of the ground leaves were extracted with 10 ml 95% ethanol under reflux at 80°C for one hour in a 20 x 2.5 cm tube connected with a 15 cm condenser. Each ethanolic extract was cooled and used directly for the analysis of plaunotol content by TLC densitometry and gas chromatography. For other *Croton* plants, sample preparations from the dried roots, stems and leaves were performed in the same manner.

2.3 Thin-Layer Chromatographic Densitometric Analysis

Five-microlitre aliquot of each ethanolic extract obtained from above was spotted on a TLC plate. The stationary phase was silica gel 60 F254 plate (Merck, Damstadt, Germany) and the mobile phase was chloroform:n-propanol which was developed three times using the solvent ratios of 24:1, 24:0.5 and 24:0.5, respectively. Plaunotol in each sample in the TLC plate was quantitated by the developed TLC densitometric method and calculated based on its standard curve. TLC conditions for plaunotol separation and densitometric analysis are described as follows.

2.3.1 Thin Layer Chromatographic Conditions for Plaunotol Separation

Technique : one way, ascending, triple development

Stationary phase : aluminium sheet silica gel 60 F254 (precoated, Merck)

Plate size : 10 x 20 cm^2

Solvent system (mobile phase) : first development:chloroform:n-propanol (24:1)
second development:chloroform:n-propanol (24:0.5)
third development:chloroform:n-propanol (24:0.5)

Sample size : 5 µl

Distance : 8 cm

Temperature : 25-30°C

Detection : Exposing with iodine in a iodine tank

2.3.2 Densitometric Analysis

Plaunotol spot obtained after thin layer chromatography was quantitated by the densitometric method as described below.

Instrumental model : Shimadsu Dual-Wavelength TLC-Scanner Model CS-930

Lamp : Deuterium (D2)

Determination mode : absorption

Scan width : X = 10.0 mm
Y = 0.05 mm

Sensitivity : medium

Slit width : 1.2 x 1.2 mm^2

Wavelength detector : 220 nm.

2.4 Gas Chromatographic Analysis

The accuracy and precision of the TLC densitometric method was evaluated using gas chromatographic method which could also be used for determining the plaunotol content in *C. sublyratus* leaves.

2.4.1 Sample Preparation for GC analysis

An aliquot of ethanolic extract (2 ml) was evaporated to dryness using Speedvac System SC 100 (Savant Instruments, Inc., USA). The residue was added with 2 ml 50% ethanol and 0.40 ml of 10% sodiumhydroxide at 70°C in a water bath for 30 min. It was cooled and extracted with 3 ml of n-hexane for three times. The hexane layers were pooled together and evaporated to dryness. The residue was dissolved in 200 µl of chloroform before injected (2.0 µl) into the GC system.

2.4.2 Gas Chromatographic Conditions

Plaunotol in *C.sublyratus* leaves was analysed by gas chromatography. The conditions of GC are described below.

GC conditions:

Instrumental model	: Varian 3400 gas chromatography equipped with 8100 Autosample (Sugar Land, Taxas, USA)
Injector model	: 1041 Universal injector
Detector	: FID
Column	: glass column (2m x 2mm) packed with 2% silicone OV 17 On 60/80 Supelcoport (Supel Co., Inc., USA)
Column temperature gradient	: initial temp = 235°C hold 2 min final temp = 250°C rate 15°C/min hold 10 min
Injector temperature	: 300°C
Detector temperature	: 300°C

Nitrogen carrier gas	: flow rate 30 ml/min
Hydrogen supply	: flow rate 30 ml/min
Air supply	: flow rate 300 ml/min
Chart speed	: 0.3 cm/min
Sample size	: 2.0 µl

2.5 Preparation of Standard Solutions for Calibration Curve

Two hundred and thirty milligrams of light yellow-brown liquid form in Kelnac capsule (Each capsule contains 80 mg of plaunotol and 150 mg of corn oil) were dissolved in 10 ml chloroform to give 0.8 mg plaunotol per ml stock solution. The stock solution was diluted to the concentration range of 0.025-0.2 mg/ml (0.125-1.0 µg/5µl) for constructing their calibration curve of plaunotol by TLC-densitometric analysis. For gas chromatography, the stock solution was diluted by half-dilution technique and the concentration range of 0.05-0.8 mg/ml (1-16 µg/2µl) was used for constructing the calibration curve of plaunotol.

3. Plant Tissue Culture Technique

3.1 Plant Material

C. sublyratus Kurz used for the tissue culture works was the high plaunotol-containing plant cultivated in the open field at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

3.2 Nutrient Media

Standard basal media used in this study included Murashige and Skoog (MS) (Murashige and Skoog, 1962) and Gamborg (B5) (Gamborg *et al.*, 1968). The composition of these media and media preparation method were described in Appendix.

Plant growth regulators used in this study are summerized as follows :

Auxin :

Indole-3-acetic acid (IAA) (Gibco Laboratories, New York, USA)

Indole-3-butyric acid (IBA) (Gibco Laboratories, New York, USA)

α-Naphthaleneacetic acid (NAA) (Gibco Laboratories, New York, USA)

2,4-Dichlorophenoxyacetic acid (2,4-D) Laboratories, New York, USA)

Cytokinin :

Kinetin-6-furfurylaminopurin (kinetin) (Gibco Laboratories, New York, USA)

6-Benzylaminopurine or N^6-Benzyladenine (BA) (Gibco Laboratories, New York, USA)

N^6-(Δ_2 Isopentenyl)-adenine (2iP) (Gibco Laboratories, New York, USA)

Gibberellin :

Gibberellic acid (GA_3) (Gibco Laboratories, New York, USA)

For solid media, the nutrient solutions were added with 0.6-0.8% (w/v) agar (Difco, Detroit Michigan, USA) or 0.2 or 0.8% (w/v) gellan gum (Sigma Chemical, MO, USA).

3.3 Culture Conditions

The *in vitro* cultures of *C. sublyratus* were maintained in a culture room with a 16-hour photoperiod (2000 lux) and 8-hour dark at 25±2°C

3.4 Preparation of *C. sublyratus* Explants

The explants of *C. sublyratus* used as starting material for plant regeneration studies included shoot tips, nodal segments with auxially buds, auxially buds, young leaves and petioles. Table 6 summarises the methods of surface sterilization and preparation of *C. sublyratus* explants.

3.5 Study on the Effect of Plant Growth Regulators on Growth and Development of *C. sublyratus* explants

Various explants including shoot tips (1.0–1.5 cm length), nodal segments (1.0–1.5 cm length) with auxially buds, auxially buds and leaf segments (0.5 x 0.5 cm^2) of *C. sublyratus* were used for this study. The effect of some basal solid media and plant growth regulators on the development of these explants were investigated. The cultures were maintained at 25±2°C under controlled photoperiod of 16 hr. The experiments for growth and development of each explant on various medium were described below.

Shoot tips. MS medium supplemented with 30 g/l sucrose and 0.8% (w/v) agar was used for the experiments. The explants were placed on MS medium containing with 2.0 mg/lBA and various different levels of auxins (IAA, IBA, NAA and 2,4-D), cytokinins (kinetin, BA and 2iP) and gibberellic acid (GA_3) at the concentrations of 0.01, 0.1, 1.0 and 10.0 mg/l.

Nodal segment with auxially buds. MS agar medium supplemented with 30 g/l sucrose, 2.0 mg/l BA were used for studying their growth and development.

Auxially buds. MS medium containing 30 g/l sucrose, 2.0 mg/l BA and 0.8% (w/v) agar was used for studying growth and development of

Table 6 Methods for the sterilization and preparation of *C. sublyratus* explants.

Explants	Procedure			
	Pre-sterilization	Sterilization	Post-sterilization	Explant preparation
Shoot tips and nodal segments with auxially buds	Young stems (ca 10 cm length) , with leaves removed were cleaned under running tap water and cut into small size (ca 5 cm length). This organs were dipped into 70% ethanol for a few second.	Sterilization in 10% Clorox solution containing a few drops of Tween 80 (a wetting agent) for 30 mins in a shaker.	Washed three times in sterile distilled water.	A piece of stem was dissected into small sections (1–1.5 cm in length) starting from the shoot tip to the nodal segments with auxillary buds. These explants were half dipped vertically into the ager media.
Auxially buds	Same as shoot tips and nodal segments with auxially buds.	Same as shoot tips and nodal segments with auxially buds.	Same as shoot tips and nodal segments with auxially buds.	Auxially buds (0.1–0.3 cm size) were removed from nodal segments and placed horizontally onto agar the media.
Leaves and petioles	Young leaves with petioles were cleand under running tap water and dipped into 70% ethanol for a few second.	Sterilization in 10% Clorox solution containing a few drops of Tween 80 for 5 mins in shaker.	Washed three times in sterile distilled water.	Leaf blade and petiole were separated befere dissected into pieces of leaf segments (0.5x0.5 cm^2). and petiole segments (ca 0.5 cm in length).Both segments were places horizontally onto the ager media.

shoot. In addition, MS medium without growth regulators, B5 medium containing 0.5 mg/l 2,4-D and 1.0 mg/l BA, 0.8% (w/v) agar and wet filter papers were used for studying shoot formation from auxially bud.

Leaf segments. Various types and concentrations of plant growth regulators in MS and B5 media were varied to study their effects on growth and development. The explants were placed on various different levels of each auxin (IAA, IBA, NAA or 2,4-D), cytokinin (kinetin, BA or 2iP) and gibberellic acid (GA_3) at the concentrations of 0.01, 0.1, 1.0 and 10.0 mg/l

All experiments were observed periodically for growth and development of each explant and the results were recorded.

3.6 Establishment of Callus Cultures

3.6.1 Callus Induction

The study of callus induction of *C. sublyratus* was carried out using two 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.

3.6.1.1 Effect of basal media

MS and B5 media supplemented with 30 g/l sucrose, 1.0 mg/l 2,4-D, 0.1 mg/l kinetin and 0.8% (w/v) agar were used for studying on callus induction of *C. sublyratus* leaf explants and petiole explants. These surface-sterilized explants were transferred onto the media and maintained at 25±2°C under controlled 16-hr photoperiod conditions.

3.6.1.2 Effect of plant growth regulators

Leaf segments used for callus induction were cultured on MS medium containing various combinations of growth regulators. 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.

3.6.2 Subculturing

The calli of *C.sublyratus* were subcultured on MS medium containing 30 g/l sucrose, 1.0 mg/l 2,4-D, 1.0 mg/l BA and 0.8% (w/v) agar for proliferation every 3 weeks and maintained at 25±2°C under light. Some callus cultures were subcultured into various media as described in the result section.

3.7 Establishment of Cell Suspension Cultures

Cell suspension cultures of *C. sublyratus* were initiated from the established callus cultures. The callus tissues were placed in a 250-ml Erlenmeyer flask containing 50 ml of MS liquid medium with 30 g/l sucrose, 0.2 mg/l NAA and 0.2 mg/l 2,4-D on a rotary shaker at 120 r.p.m. at 25±2°C. These cell suspensions were subcultured on the same medium every 4 weeks by adding 10 ml of culture to 50 ml to fresh medium.

3.8 Shoot Regeneration from Callus Cultures

The study of shoot regeneration of *C. sublyratus* from its callus was carried out in three stages : callus induction from leaf explants, green callus formation and proliferation, and shoot formation via callus.

3.8.1 Callus Induction

Callus cultures of *C. sublyratus* were first initiated from young leaf explants by culturing on callus induction medium (see section 3.6.1.2). The best callus growth was obtained two weeks later in MS medium containing 30 g/l sucrose, 2.0 mg/l 2,4-D, 1.0 mg/l kinetin and 0.8% (w/v) agar at 25±2°C under 16-hr photoperiod of fluorescent light.

3.8.2 Green Callus Formation and Proliferation

After callus induction, the calli were transferred onto MS medium containing with 10 g/l sucrose, 2.0 mg/l NAA, 2.0 mg/l BA and 0.2% (w/v) gellan gum for green callus formation and proliferation by subculturing every 3 weeks on this medium under the same condition as callus induction. The green callus was used for the shoot formation.

3.8.3 Shoot Formation via Callus

Green callus of *C. sublyratus* was cultured on MS solid medium containing 10 g/l sucrose, 1.0 mg/l BA and 0.05 mg/l GA_3 for adventitious shoot formation. The media were solidified with either 0.6% (w/v) agar or 0.2% (w/v) gellan gum. The cultures were maintained in the dark for one week and then transferred to incubate under 16-hr photoperiod of fluorescent light at 25±2°C.

In addition, the callus cultures were grown on the medium described in sections 3.6.2 and 3.8.1 in order to study the effect of various media on shoot formation. These media included : 1) MS solid medium containing 10 g/l sucrose, 1.0 mg/l BA and 0.05 mg/l GA_3, and 2) MS solid medium containing 10 g/l sucrose, 2.0 mg/l BA and 0.5 mg/l GA_3. The

media were solidified with either 0.6% (w/v) agar or 0.2% (w/v) gellan gum. The cultures were maintained under the same conditions as described above.

At least 10 replicates were used and each experiment was repeated twice. The growth and development of callus in each medium were observed periodically and the results were recorded.

3.9 Root Induction

The regenerated shoots obtained as described in section 3.8.3, were transferred onto the media for rooting at 25±2°C under 16-hr photoperiod of fluorescent light. The culture medium used for study on root induction was MS containing 10 g/l sucrose and 0.2% (w/v) gellan gum.