CHAPTER 3

MATERIALS AND METHODS

3.1 Secondary compound production from root cultures of *Stemona curtisii*

3.1.1 Plant materials and multiple shoot induction

Buds adjacent to the tuberous roots of *Stemona curtisii* Hook. f. were obtained from Trang Province, Southern Thailand. They were washed under running tap water and surface sterilized by shaking for 5 min in 0.1% mercuric chloride solution (w/v). Buds were then washed three times in sterile distilled water and placed on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 2 mg/L N6-benzyladenine (BA) and 3% (w/v) sucrose and solidified with 0.2% (w/v) gelrite (Sigma-Aldrich, USA). The pH of the medium was adjusted to 5.8 with 0.1 M KOH before autoclaving at 121 ˚C for 15 min. Cultures were incubated at 25±2 °C under 16 h/d photoperiods. Multiple shoots were induced from the buds explants after 2-3 months of culturing, they showed extensive proliferation. *In vitro* shoot explants were placed on the root culture medium, MS medium supplemented with 1 mg/L naphthalene acetic acid (NAA) and solidified with 0.2% (w/v) gelrite at 25±2°C, under 16 h/d photoperiods. Roots were generated after culturing for 3 months.

3.1.2 Time profile study of alkaloid formation in root culture

In vitro roots of *Stemona curtisii* Hook. F were transferred to semi-solid MS medium supplemented with 1 mg/L NAA. They were cultured at 25 °C and 16 h/d photoperiods for 24 weeks and harvested every 4 weeks. Root

and medium were extracted for *Stemona* alkaloids and analyzed by high performance liquid chromatography (HPLC).

3.1.3 Effects of elicitors on growth and *Stemona* alkaloids production in root cultures

1 g Chitosan (Sigma-Aldrich, Germany) was dissolved in 1,000 ml of 1% acetic acid and the pH of the solution was adjusted to 5.8 using 0.1 M KOH before added into the medium at final concentration 0, 1, 5 and 10 mg/L. Yeast extract (Lab-scan, Thailand, 0.5, 1, 2 and 5 g/L) or salicylic acid (SA) (Sigma-Aldrich, Germany, 100, 200, 300, and 500 mg/L) was added to the MS medium at an appropriate concentration and the pH was adjusted to pH 5.8 before autoclaving. Methyl jasmonate (MJ) (Sigma-Aldrich, Germany) was filter-sterilized before its addition to the sterile MS medium at final concentration 100, 200, 300 and 500 μM. Roots (1 g fresh weight) were inoculated into elicitation medium and control medium (without elicitor addition) for 16 weeks at 25 °C, with 16 h photoperiod (2000 lux, cool white fluorescent tubes).

3.1.4 Effects of precursors on growth and *Stemona* alkaloids production in root cultures

The roots (1 g fresh weight) were cultured for 16 weeks on semi-solid MS medium containing different concentrations of precursors: sucrose (4, 5, 6 and 7% (w/v)), L-tyrosine (Fluka, Switzerland, 10, 20 30 and 50 mg/L) or sodium acetate (Merck, Germany, 20, 50 and 100 mg/L). Precursors were added to the semi-solid MS medium and the pH was adjusted to 5.8 before autoclaving. MS medium without the precursors was the control medium. Since the growth and *Stemona* alkaloids content of roots reached maximum on the 16th week of culturing, root growth (fresh weight, dry weight and growth

index) and stemocurtisine, stemocurtisinol and oxyprotostemonine content were measured after 16 weeks of culturing.

3.1.5 Effects of culture condition on growth and *Stemona* alkaloids production in root cultures

Roots (1 g fresh weight) of *Stemona curtisii* Hook. f were cultured under different conditions: medium pH (5.0, 5.5 and 5.8), culture temperature at 20, 25 and 30 °C in a Growth Chamber (Model 620RHS -400L, Contherm scientific limited, Australia) with altered lighting conditions (dark or under illumination with light intensity of 2,000 Lux 16 h /day). Root growth (fresh weight and dry weight) and stemocurtisine, stemocurtisinol and oxyprotostemonine content were measured after 16 weeks of culturing.

3.1.6 Root fresh and dry weight (dw) determination

Roots were collected and the fresh weight was measured. For dry weight determination, the roots were dried with hot air oven (venticell) at 35 °C. After total elimination of water was achieved, the dried roots were weighed and the alkaloid content was analyzed by HPLC.

3.1.7 Alkaloid extraction

3.1.7.1 Root extract

Dried root powder (1 g) was macerated sequentially with 3 x 50 ml methanol (Merck, HPLC grade, Germany) at room temperature over 3 days. The methanol solution (150 ml) was filtered and evaporated at 35 °C. Then the crude methanol extract was dissolved in 1 ml methanol and 1 ml water before extraction with dichloromethane (3 x 5 ml) to give the partially purified extract after evaporation of the

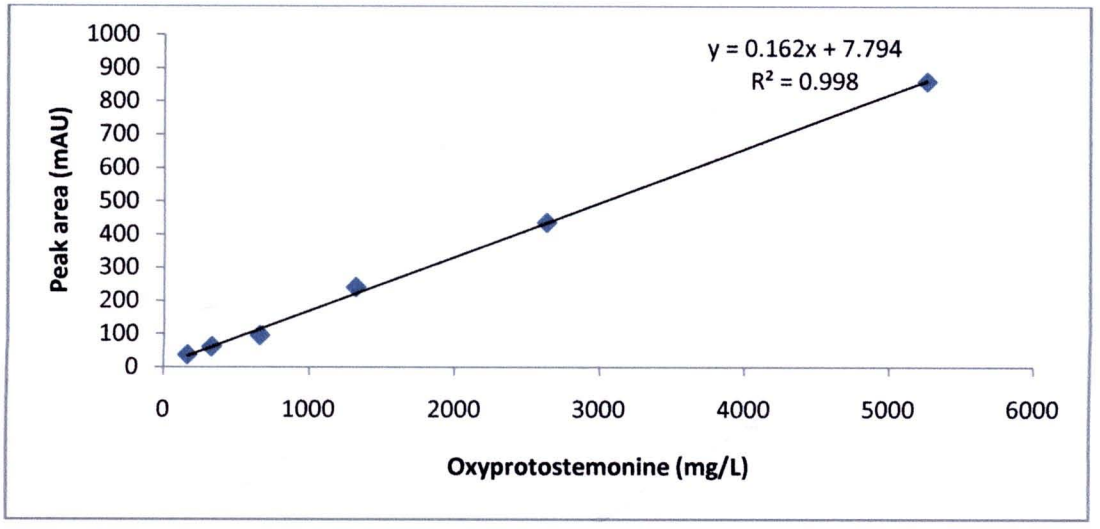
solvent. The weight was recorded and the extract was analyzed by HPLC.

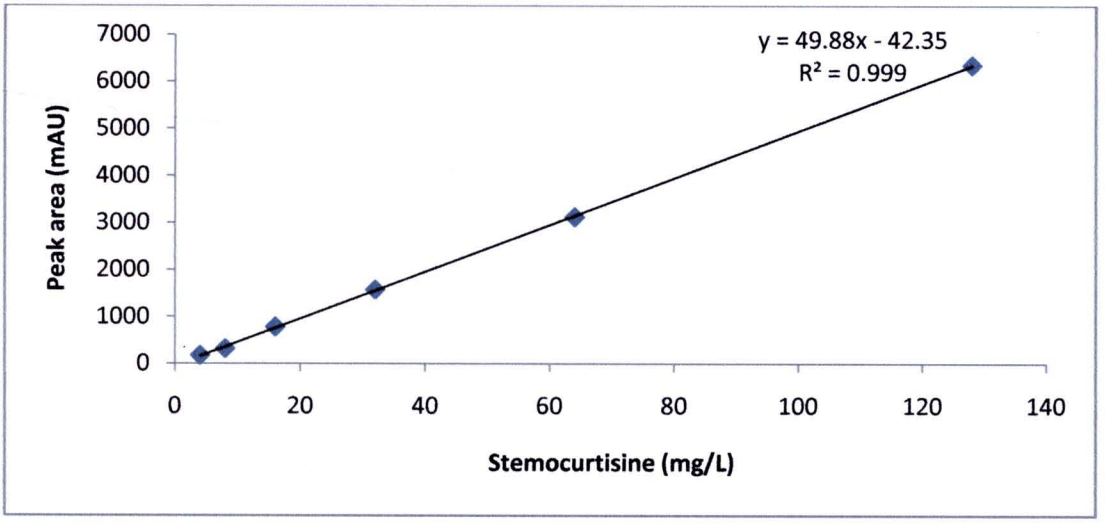
3.1.7.2 Medium extract

The medium was extracted with dichloromethane (Merck, HPLC grade, Germany) at a ratio of medium: dichloromethane of 1:1 (3 times). The dichloromethane fraction was separated and filtered before evaporated at 35 °C to give the partially purified extract. The weight was recorded and the extract was analyzed by HPLC.

3.1.8 High-Performance Liquid Chromatography (HPLC) Analysis of Alkaloids from the Roots and the Exudates

Quantification was based on the external standard method using calibration curves (Figure 3.1). The mixed working standard solutions containing oxyprotostemonine, stemocurtisine and stemocurtisinol in the concentration range of 164-5,258, 4-128 and 0.625-20 mg/L were prepared, respectively (Table 3.1). The analysis of these compounds were performed using an Agilent 1100 HPLC system equipped with UV detector at wavelength of 297 nm (Agilent Technologies, Palo Atto, CA, USA). 20 µL of solution was injected onto reversed phase (Inertsil ODS-3, 5 µm, 4.6 I.D. x 150 mm, GL Sciences Inc., Japan). HPLC column and eluted at flow rate 1.0 mL/min with methanol (Merck, HPLC grade, Germany)-Milli-Q water (60:40, v/v). Prior to the next run, the HPLC column was equilibrated further for 30 min. Data acquisition and analysis were performed by the Agilent ChemStation software. The retention times of oxyprotostemonine, stemocurtisine and stemocurtisinol were 2.37, 4.15 and 7.67 min, respectively (Figure 3.2).





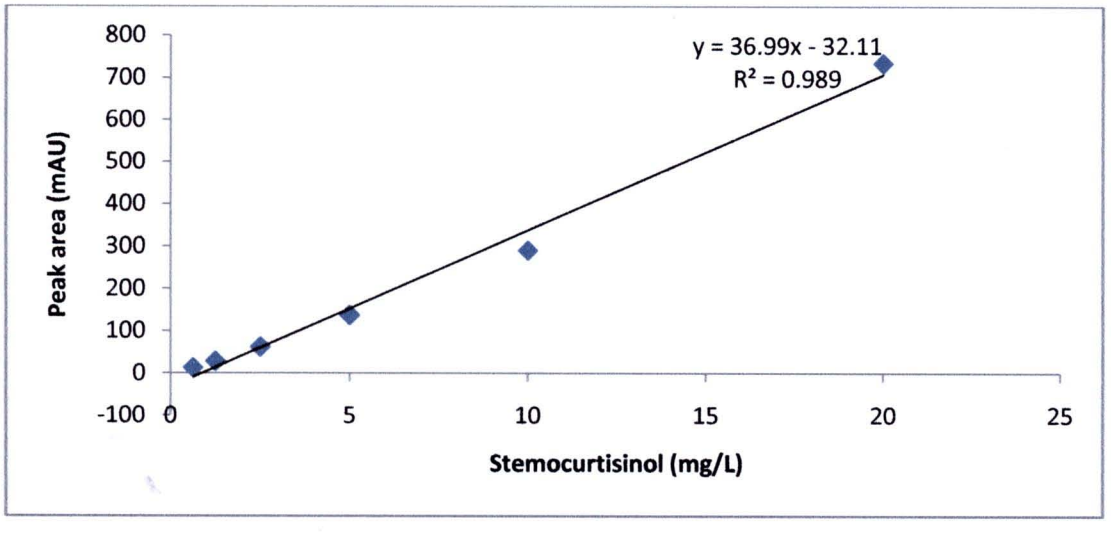


Figure 3.1 Calibration curves of oxyprotostemonine, stemocurtisine and stemocurtisinol standards that described in Table 3.1

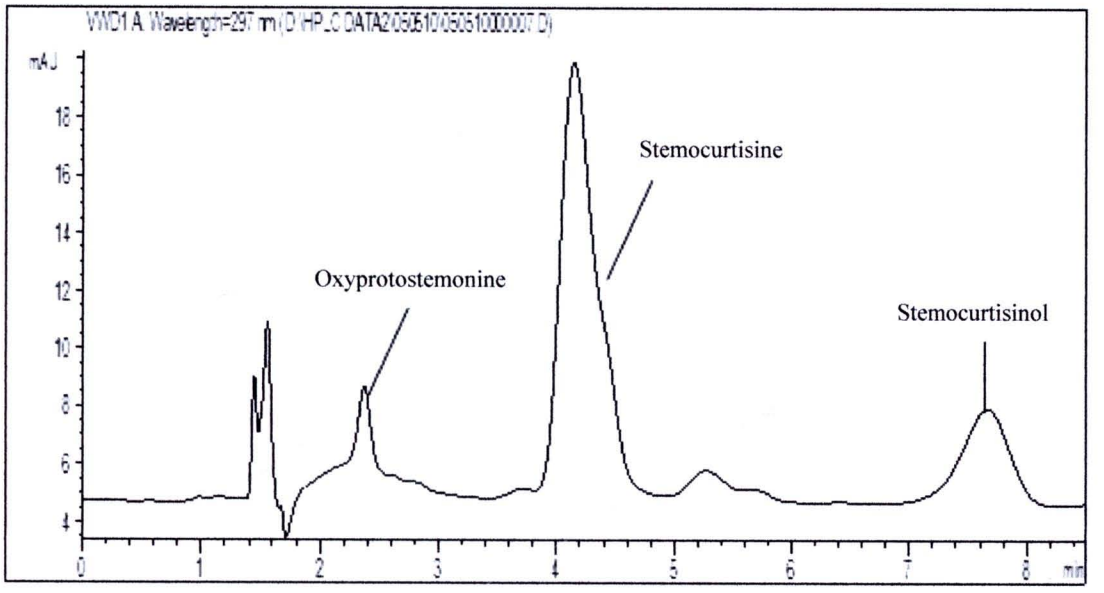


Figure 3.2 Chromatogram of a standard mixtures of oxyprotostemonine (164.3 mg/L), stemocurtisine (4 mg/L) and stemocurtisinol (0.625 mg/L)

3.1.9 Limitation of the analysis

Stemona alkaloid standards were not from a commercial source. They were isolated from the root extracts of *S. curtisii* growing in the northern part of Trang Province in Thailand by Dr. Pitchaya Mungkornasawakul. Standards of oxyprotostemonine, stemocurtisine and stemocurtisinol were analyzed for their purities by HPLC analysis (3 samples/standard). The purities of oxyprotostemonine, stemocurtisine and stemocurtisinol were, 79.4, 65.5 and 82.0% based on peak area, respectively.

3.1.10 Statistical analysis

All experiments were repeated at least thrice with 15 replicates per treatment. All the values are expressed as the mean±SE. The data were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered statistically significant.

Table 3.1 Concentration of oxyprotostemonine, stemocurtisine and stemocurtisinol standards versus HPLC peak areas.

Oxyprotostemonine (mg/L)	Peak area (mAU*s)	±Standard Error	Stemocurtisine (mg/L)	Peak area (mAU*s)	±Standard Error	Stemocurtisinol (mg/L)	Peak area (mAU*s)	±Standard Error
164.3	37.32	1.86	4	173.60	13.89	0.625	13.15	0.66
328.6	61.08	3.09	8	317.09	25.36	1.250	28.11	1.42
657.2	94.89	4.84	16	781.79	62.54	2.500	61.76	3.09
1314.5	241.00	12.11	32	1570.82	125.66	5.000	137.17	6.89
2629.0	436.63	21.91	64	3121.32	249.70	10.000	289.95	14.51
5258.0	860.80	43.05	128	6351.14	508.09	20.000	734.02	36.67

3.2 Secondary compound production from root cultures of *Stemona* sp.

3.2.1 Plant materials and culture medium

Stemona sp. was originally obtained from Amphur Mae Moh, Lampang province, Northern Thailand. Shoot tips and axillary buds were surface sterilized with 15% clorox solution for 20 minutes followed by 3 times washing with sterile distilled water. They were then cultured on MS medium supplemented with 3 mg/L BA and 3% sucrose. Medium pH was adjusted to 5.8 with 1M KOH and solidified with 2% (w/v) Gelrite (Sigma-Aldrich, USA) before autoclaving at 121 ˚C for 15 min. The cultures were cultured at 25± 2 °C under 16 h/d photoperiod. Multiple shoots were induced 8 weeks of culturing, they showed extensive proliferation. *In vitro* shoot explants were placed on the root induction medium, 1/2MS medium supplemented with 2 mg/L IBA, 3% sucrose and solidified with 0.2% (w/v) gelrite at 25±2°C, under 16 h/d photoperiods. Roots were generated after culturing for 8 weeks.

3.2.2 Time profile study of 1′,2′-didehydrostemofoline production in root cultures of *Stemona* sp.

In vitro roots of *Stemona* sp. (1 g fresh weight) were transferred to semi-solid half-MS medium supplemented with 2 mg/L IBA, 3% (w/v) sucrose and solidified with 0.2% (w/v) gelrite. The pH of the medium was adjusted to 5.8 with 0.1 M KOH before autoclaving at 121 ˚C for 15 min. Cultures were incubated at 25±2 °C under 16 h/d photoperiods for 24 weeks and harvested every 4 weeks. Roots were extracted and analyzed by HPLC.

3.2.3 Root extraction method and HPLC condition

Dry roots (1 g) of *Stemona* sp. were ground and extracted 3 times with 50 mL methanol (Merck, HPLC grade, Germany). The solution was filtered

and evaporated to get crude extract which was extracted again with dichloromethane (DCM) (Merck, HPLC grade Germany). The extract was concentrated to get crude DCM extracts and the weight of crude extracts was also recorded. The crude DCM extract was dissolved in methanol and filtered with 0.45 μM membrane filter (Filtrex syringe membrane filtration). Quantification was based on the external standard method using calibration curves (Figure 3.3). The standard solutions of 1′,2′-didehydrostemofoline in the concentration range of 62.5-2,000 ng/mL were prepared (Table 3.2). The analysis of 1′,2′-didehydrostemofoline was performed using an Agilent 1200 HPLC system equipped with UV detector at wavelength of 297 nm (Agilent Technologies, Palo Atto, CA, USA). 20 μL of solution was injected onto reversed phased (Inertsil ODS-3, 5 μm, 4.6 I.D. x 150 mm, GL Sciences Inc., Japan). HPLC column and eluted at flow rate 0.3 mL/min with methanol (Merck, HPLC grade, Germany)-Milli-Q water (90:10, v/v). Prior to the next run, the HPLC column was equilibrated further for 15 min. Data acquisition and analysis were performed by the Agilent ChemStation software. The retention times of 1′,2′-didehydrostemofoline was 7.945 min, (Figure 3.4).

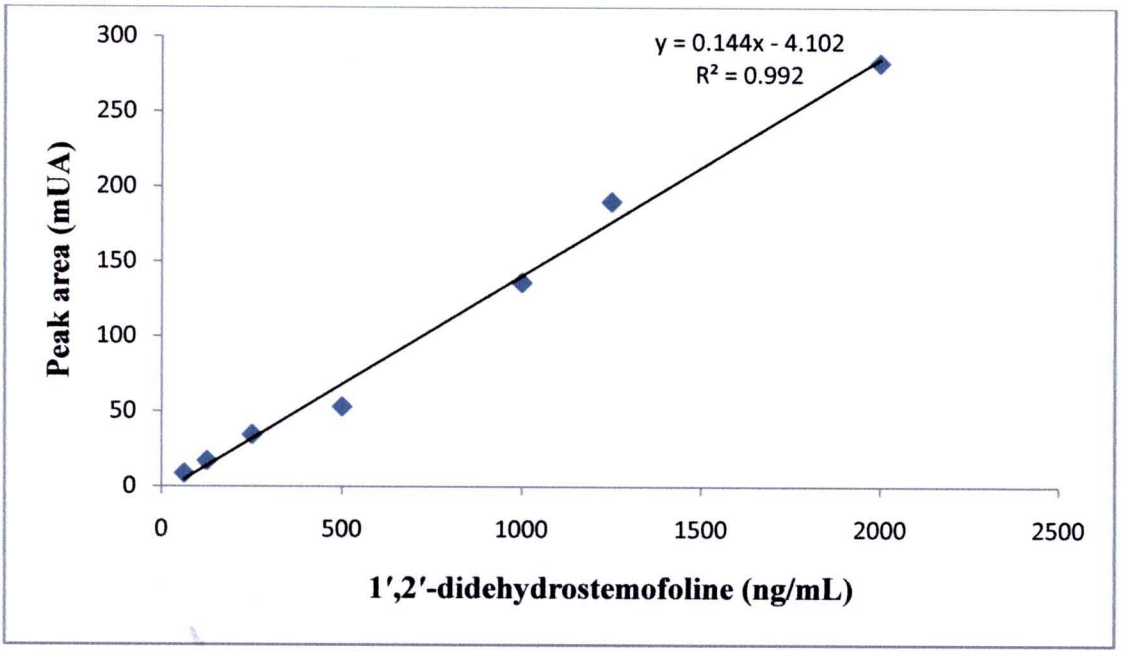


Figure 3.3 Calibration curve of a standard 1′,2′-didehydrostemofoline Standard that described in Table 3.2

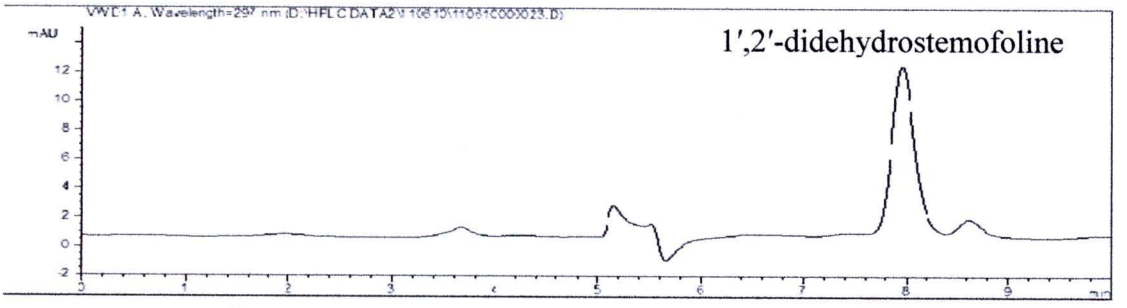


Figure 3.4 Chromatogram of a standard 1′,2′-didehydrostemofoline.

Table 3.2 Concentration 1′,2′-didehydrostemofoline standard versus HPLC peak area.

1′,2′-didehydrostemofoline (ng/mL)	Peak area (mAU*s)
62.5	8.71
125	17.07
250	34.60
500	53.12
1000	135.94
1250	190.19
2000	282.63

3.2.4 Statistical analysis

All experiments were repeated at least thrice with 15 replicates per treatment. All the values are expressed as the mean±SE. The data were analyzed by using one-way analysis of variance (ANOVA) followed by Tukey's test. $P<0.05$ was considered statistically significant.