CHAPTER III

EXPERIMENTAL

1. Source of Plant Material

The stems of *Strychnos vanprukii* Craib (Loganiaceae) were collected from Chaiyapum province, Thailand in July, 1998. The plant material was authenticated by comparison with the herbarium specimen at Royal Forest Department, Bangkok, Thailand.

A voucher specimen of the plant material has been deposited in the herbarium of the Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

2. General Techniques

2.1 Chromatography

2.1.1 Analytical Thin-layer Chromatography (TLC)

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Technique : One dimension, ascending

Adsorbent : Silica gel 60 F254 (E. Merck) precoated plate

Layer thickness : 0.2 mm

Distance : 7 cm

Temperature : Laboratory temperature (30-35 °C)

Detection :
1). Visual detection under daylight
2). UV light at the wavelengths of 254 and 365 nm
3). Spraying with vanillin-sulphuric acid reagent (10% ethanolic sulphuric acid) and heating at 110 °C for 5-10 minutes

4) Spraying with ferric chloride-perchloric acid

The reagent was made by mixing 1 ml. of 0.5 M ferric chloride solution with 100 ml. of 35% aqueous perchloric acid solution.

The reagent gave a variety of colors depending on the nature of the substitution pattern in the aromatic part of the *N*-acylindoline nucleus and also on different type of alkaloid skeletons. The color would either develop immediately after spraying or only after heating the chromatographic plate at 90° C for 5-30 minutes.

5) Spraying with Dragendorff Reagent

This reagent was used as general alkaloidal detecting reagent which characterized the alkaloids by giving orange color. The stock solution consisted of 1.7 g bismuth oxynitrate, 20 ml of glacial acetic acid, 80 ml of distilled water and 100 ml of 5% aqueous potassium iodide.

The working solution was made by mixing 10 ml. of the stock solution with 20 ml. of glacial acetic acid and 70 ml. of distilled water.

2.1.2 Column Chromatography

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Adsorbent : silica gel 60 G (No. 9385 E. Merck).

Packing method : wet packing and dry packing

Solvent : various solvent systems depending on the material to be separated.

2.1.3 Gel Filtration Chromatography

Gel filter : Sephadex LH-20 (Pharmacia)

Packing method	:	The gel filter was suspended in the eluent and left standing to swell for 24 hours prior to use. It was then poured into the column and allowed to set tightly.
Sample loading	:	The sample was dissolved in a small volume of the eluent and applied onto the top of the column.

2.2 Spectroscopy

2.2.1 Ultraviolet (UV) Absorption Spectra

UV spectra (in methanol) were obtained on a Milton Roy Spectronic 3000 Array spectrometer (Pharmaceutical Research Equipment Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.2.2 Infrared (IR) Absorption Spectra

IR spectra (KBr disc or dissolved in methanol) were obtained either on a Perkin Elmer FT-IR 2000 spectrometer or a Perkin Elmer FT-IR 1760X spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

2.2.3 Mass Spectra (MS)

Electron Impact Mass Spectra (EIMS) were recorded on a Polaris Q mass spectrometer (Faculty of Science, Mahidol University). Electron Spray Ionization Mass Spectra were recorded on an Applied Biosystems Q-STAR (quadrupole-tof) mass spectrometer (Gorlaeus Laboratory, Leiden University).

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2.2.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

^{1}H-NMR (300 MHz) and ^{13}C-NMR (75 MHz) spectra were obtained with a Bruker Avance DPX-300 FT-NMR spectrometer (Faculty of Pharmaceutical Sciences, Chulalongkorn University and Gorlaeus Laboratory, Leiden University).

^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were obtained with a JEOL JMN-A 500 NMR spectrometer (Scientific and Technological

Research Equipment Center, Chulalongkorn University). Chemical shifts were reported in ppm scale using the chemical shift of solvents as the reference signal.

2.3 Physical Properties

2.3.1 Melting Points

Melting points were obtained on a Fisher/Johns Melting Point Apparatus (Department of Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Chulalongkorn University) and are uncorrected.

2.3.2 Optical rotation

Optical rotation was measured on a Perkin Elmer 341 polarimeter (Pharmaceutical Research Equipment Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

2.4 Solvents

Throughout this work, all organic solvents used were commercial grade and had to be redistilled prior to use.

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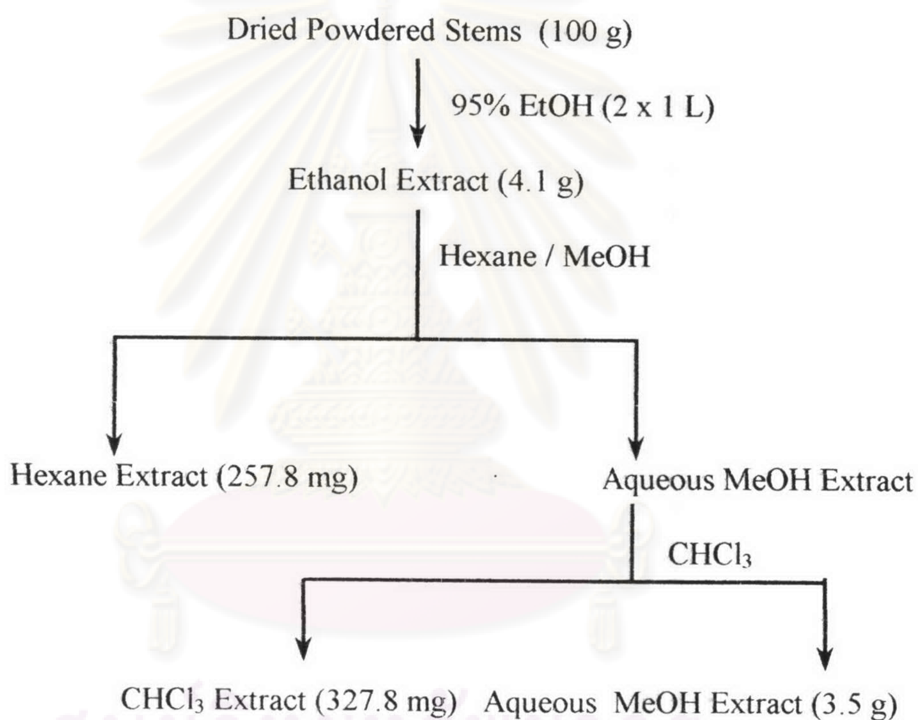
3. Extraction Procedure

The dried stems of *Strychnos vanprukii* Craib. (100 g) were blended into small pieces. They were extracted twice with 95% ethanol (1 L, 3 days each). The filtrates were pooled and evaporated under reduced pressure at temperature not exceeding 50 °C to yield the ethanol extract as a syrupy mass (4.1 g) and insoluble powder (3.1 g). The extract was dissolved in aqueous MeOH and partitioned with hexane followed by $CHCl_3$ to obtain hexane (257.8 mg), $CHCl_3$ (327.8 mg) and aqueous MeOH extract (3.5 g).



Scheme 13 Extraction of *Strychnos vanprukii* Craib Stem.

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4. Isolation Procedure

The methanol extract (3.5 g) was subjected to silica gel column chromatography, using the mixture of chloroform - methanol (4 : 1) as the eluent. The extract was dissolved in methanol and mixed with kieselguhr, then applied to the top of a glass column (3 x 45 cm) already packed with a slurry of silica gel (100 g) in the eluting solvent. Fractions (1 ml each) were monitored by TLC, with chloroform – methanol (7 : 3) and ethyl acetate-isopropanol-concNH_4OH (45 : 35 : 20) as the developing solvent system. A total of 1593 were collected and combined according to their TLC profiles into six major fractions (M1 – M6) as shown in Table 5. The column was then washed down with methanol and the next 207 fractions were combined as fraction M7.

Table 5 Combined fractions from the methanol extract of *Strychnos vanprukii* stems

Code	Number of fractions	Weight of combined fraction (mg)
M1	1-22	54.8
M2	23-41	98.7
M3	42-120	132.4
M4	121-470	1086.5
M5	471-800	214.4
M6	800-1593	295.7
M7	1594-1800	1382.7

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4.1 Isolation of compound SVM 1

Fraction M2 was subjected to silica gel column chromatography. The fraction (98.7 mg) was dissolved in a small volume of chloroform-acetone-methanol (10 : 2 : 3) and applied to the top of a glass column (1.8 x 21.5 cm) already packed with a slurry of silica gel (12 g). This same solvent mixture was employed as the

eluting solvent. The fraction volume collected was about 1 ml each. All eluates were collected and combined according to their TLC patterns, using the eluting solvent as the developing solvent system. Fraction M2 was further separated into four fractions as summarized in Table 6.

Table 6 Combined fractions from fraction M2

Number of fractions	Weight of combined fraction (mg)
1-22	15.9
23-28	23.8
29-45	22.3
46-80	31.7

Compound SVM-1 (23.8 mg) was obtained as amorphous powder from fractions 23-28.

4.2 Isolation of compound SVM 2

Fraction M3 (132.4 mg) was purified by washing with methanol to give compound SVM-2 which appeared as cubic crystals (5.8 mg).

4.3 Isolation of compound SVM 3

After repeated recrystallization in methanol, fraction M4 (1086.5 mg) yielded compound SVM-3 as colorless needles with the combined weight of 712.8 mg.

4.4 Isolation of compound SVM 4

Fraction M5 (214.4 mg) was purified by recrystallizing in methanol-acetone to afford compound SVM-4 as amorphous powder (35.8 mg).

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4.5 Isolation of compound SVM 5 and SVM 6

Fraction M6 (295.7 mg) was separated by a silica gel column (12 g, 2 x 18.5 cm) with chloroform-methanol (6 : 5) as the mobile phase. The fractional volume was about 1 ml each. Ethyl acetate-isopropanol-concNH_4OH (45 : 35 : 20) was used as the developing solvent system for TLC monitoring of these fractions. Two hundred and fifty fractions were collected and then combined into 5 major fractions as summarized in Table 7.

Table 7 Combined fractions from fraction M6

Number of eluates	Weight of combined fraction (mg)
1-97	79.3
98-109	8.5
110-141	53.2
142-163	15.8
164-250	107.4

Fractions 98-109, which displayed an intense blue fluorescence on TLC under UV light, were combined and yielded compound SVM 5 (8.5 mg) as yellow amorphous powder. Compound SVM 6 which showed green fluorescence on TLC under UV light was obtained as greenish-yellow amorphous powder from fractions 142-163.

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4.6 Isolation of compounds SVC 1, SVC 2 and SVC 3

The chloroform extract (327.8 mg) was subjected to silica gel column chromatography, using the mixture of chloroform - methanol (10 : 1) as the eluent. The extract was dissolved in a small volume of the eluent and applied to the top of a glass column (1.8 x 21.5 cm) already packed with a slurry of silica gel (12 g) in the eluting solvent. Fractions (1 ml each) were monitored by TLC, with chloroform – methanol (10 : 1) and chloroform-acetone-methanol (10 : 2 : 3) as the developing

solvent system. About 130 fractions were collected and combined according to their TLC profiles into five major fractions (C1-C5) as shown in Table 8.

Table 8 Combined fractions from the chloroform extract of *Strychnos vanprukii* stems

Code	Number of fractions	Weight of combined fraction (mg)
C1	1-16	21.7
C2	17-31	85.2
C3	32-47	37.5
C4	48-73	74.6
C5	74-130	86.4

Fraction C2 (85.2 mg) was purified by washing with methanol to give compound SVC-1 as colorless needles (23.7 mg). The rest of fraction C2 was subjected to a silica gel column. The fraction (61.5 mg) was dissolved in a small volume of chloroform-methanol (10 : 1) and applied to the top of a glass column (2 x 18.5 cm) already packed with a slurry of silica gel (12 g). This same solvent mixture was employed as the eluting solvent. The fraction volume collected was about 1 ml each. All eluates were collected and combined according to their TLC patterns, using eluting solvent as the developing solvent system. Fraction C2 was thus further separated into seven fractions as summarized in Table 9.

Table 9 Combined fractions from fraction C2

Number of eluates	Weight of combined fraction (mg)
1-6	10.8
7-8	2.8
9-15	9.5
16-18	1.2
19-28	15.7
29-32	5.3
33-50	13.4

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Compounds SVC 2 and SVC 3 were obtained as amorphous powder from fractions 16-18 and 29-32, respectively.

4.7 Isolation of compounds SVC 4 and SVC 5

Fraction C3 (37.5 mg) was subjected to silica gel chromatography (9 g, 2 x 18.5 cm), which resulted in the isolation of compound SVC 4 (7.1 mg) and compound SVC 5 (8.8 mg) by using chloroform-methanol-2% acetic acid (10 :1:0.1) as eluting solvent. The fractional volume was about 1 ml each. All eluates were collected and combined according to their TLC patterns, using eluting solvent as the developing solvent system. Fraction C3 was further separated into five portions as summarized in Table 10.

Table 10 Combined fractions from fraction C3

Number of eluates	Weight of combined fraction (mg)
1-11	4.9
12-15	7.1
16-24	5.7
25-28	8.8
29-45	9.2

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4.8 Isolation of compounds SVC-6 and SVC-7

Fraction C4 (74.6 mg) was separated by a silica gel column (10 g, 1.8 x 21.5 cm) with chloroform-acetone-methanol (10 : 2 : 3) as the mobile phase. The fractional volume was about 1 ml each. Eluting solvent was used as the mobile phase for TLC monitoring of these fractions. Eighty-three fractions were collected and then combined into 7 major ones as summarized in Table 11.

Table 11 Combined fractions from fraction C4

Number of eluates	Weight of combined fraction (mg)
1-24	11.7
25-32	15.3
33-37	5.6
38-45	14.8
46-55	7.9
56-63	5.3
64-83	10.2

Fractions 25-32 and 38-45 yielded compounds SVC-6 (15.3 mg) and SVC-7 (14.8 mg), respectively, as amorphous powder.

4.9 Isolation of compounds SVH 1 and SVH-2

The hexane extract (258.7 mg) was subjected to silica gel column chromatography, using the mixture of chloroform - hexane (3 : 1) as the eluent. The extract was dissolved in a small volume of the eluent and applied to the top of a glass column (1.8 x 21.5 cm) already packed with a slurry of silica gel (13 g) in the eluting solvent. Fractions (1 ml each) were monitored by TLC, with chloroform – hexane (3 : 1) and chloroform as the developing solvent system. Eighty-five fractions were collected and combined according to their TLC profiles into five major fractions as shown in Table 12.

Table 12 Combined fractions from the hexane extract of *Strychnos vanprukii* stems

Code	Number of fractions	Weight of combined fraction (mg)
H1	1-26	35.4
H2	27-36	137.6
H3	37-55	21.8
H4	56-60	5.2
H5	61-85	52.9

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Fraction H2 (137.6 mg) was purified by washing with methanol to give compound SVH-1 as colorless needles (108.7 mg). Evaporation of the washing solvent gave compound SVH 2 (28.9 mg) as yellowish viscous oil.

4.10 Isolation of compound SVH-3

Fraction H3 was subjected to silica gel column chromatography. The fraction (21.8 mg) was dissolved in a small volume of ethyl acetate-hexane (5 : 2) and applied to the top of a glass column (2 x 18.5 cm) already packed with a slurry of silica gel (8 g). This same solvent mixture was employed as the eluting solvent. The fraction volume collected was about 1 ml each. All eluates were collected and combined according to their TLC patterns, using the eluting solvent as the developing solvent system. Fraction H3 was further separated into three fractions as summarized in Table 13.

Table 13 Combined fractions from fraction H3

Number of eluates	Weight of combined fraction (mg)
1-9	5.1
10-18	4.3
19-37	10.7

Compound SVH-3 (3.2 mg) was obtained as orange needles from fractions 10-18 by recrystrallization in methanol.

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5. Physical and spectral data of isolated compounds

5.1 Compound SVM-1

Compound **SVM-1** was obtained as amorphous powder, soluble in methanol.

Melting point	: 178-180 °
UV λ_{max} (MeOH), nm (log ε); Figure 2	: 205 (3.94), 280 (2.79)
IR ν_{max} (MeOH), cm^{-1} ; Figure 3	: 3366, 2938, 1613, 1461, 1218, 1112, 1070, 1033
EIMS *m/z* (% relative intensity); Figure 4	: 582 (M^+, 34), 421 (15), 420 (61), 402 (100), 401 (86), 371 (72), 369 (40)
^{1}**H-NMR** (δ ppm, 300 MHz, CD_3OD)	: Figure 5, Table 14
^{13}**C-NMR** (δ ppm, 75 MHz, CD_3OD)	: Figure 6, Table 14

5.2 Compound SVM-2

Compound **SVM-2** was obtained as cubic crystals, soluble in water and DMSO.

Melting point	: 201-203 °
UV λ_{max} (MeOH), nm (log ε); Figure 13	: 202 (2.80)
IR ν_{max} , (KBr) cm^{-1} ; Figure 14	: 3411, 3327, 1131, 1074, 1028, 927, 890, 725
^{1}**H-NMR** (δ ppm, 500 MHz, DMSO-d_6)	: Figures 15-16, Table 15
^{13}**C-NMR** (δ ppm, 125 MHz, DMSO-d_6)	: Figure 17, Table 15

5.3 Compound SVM-3

Compound **SVM-3** was obtained as colorless needles, soluble in methanol.

Melting point	: 206-208°
UV λ_{max} (MeOH), nm (log ε); Figure 26	: 220 (4.11), 272 (3.37)
IR ν_{max} (KBr), cm^{-1} ; Figure 27	: 3406, 1643, 1553, 1454, 1386, 1192, 1072, 996, 748
ESMSMS *m/z* (positive ion mode);	: 531 $[M+H]^+$, 369

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Figure 28	
^{1}H-NMR (δ ppm, 500 MHz, DMSO-d_6)	: Figure 29-31, Table16
^{13}C-NMR (δ ppm, 125 MHz, DMSO-d_6)	: Figure 32, Table 16

5.4 Compound SVM-4

Compound **SVM-4** was obtained as amorphous powder, soluble in methanol.

Melting point	: 129-131°
UV λ_{max} (MeOH), nm (log ε); Figure 44	: 207 (3.75), 303 (2.46)
IR ν_{max} (MeOH), cm^{-1} ; Figure 45	: 3354, 2938, 1632, 1585
ESMSMS *m/z* (positive ion mode); Figure 46	: 317 [M+H]$^+$
^{1}H-NMR (δ ppm, 300 MHz, CD_3OD)	: Figure 47, Table 17
^{13}C-NMR (δ ppm, 75 MHz, CD_3OD)	: Figure 48, Table 17

5.5 Compound SVM-5

Compound **SVM-5** was obtained as yellowish amorphous powder, soluble in methanol.

Melting point	: 216-218°
UV λ_{max} (MeOH), nm (log ε); Figure 55	: 204 (4.05), 253 (3.59), 309 (3.40), 373 (2.69)
IR ν_{max} (MeOH), cm^{-1} ; Figure 56	: 3401, 2926, 1670, 1635, 1528, 1503, 1385, 1201, 1157, 1076
ESMSMS *m/z* (positive ion mode); Figure 57	: 527 [M]$^+$, 365
^{1}H-NMR (δ ppm, 300 MHz, CD_3OD)	: Figure 58, Table 18
^{13}C-NMR (δ ppm, 75 MHz, CD_3OD)	: Figure 59, Table 18

5.6 Compound SVM-6

Compound **SVM-6** was obtained as greenish-yellow amorphous powder, soluble in methanol.

Melting point	: 203-204°

UV λ_{max} (MeOH), nm (log ε); Figure 60	: 209 (4.00), 360 (3.70)
IR ν_{max} (MeOH), cm^{-1} ; Figure 61	: 3367, 2925, 1636, 1581, 1394, 1075
ESMSMS *m/z* (positive ion mode); Figure 62	: 529 $[M]^+$, 367
^{1}H-NMR (δ ppm, 300 MHz, CD_3OD)	: Figure 63, Table 19
^{13}C-NMR (δ ppm, 75 MHz, CD_3OD)	: Figure 64, Table 19

5.7 Compound SVC-1

Compound **SVC-1** was obtained as colorless needles, soluble in DMSO.

^{1}H-NMR (δ ppm, 300 MHz, DMSO-d_6)	: Figure 74

5.8 Compound SVC-2

Compound **SVC-2** was obtained as amorphous powder, soluble in chloroform.

Melting point	: 187-189°
UV λ_{max} (MeOH), nm (log ε); Figure 75	: 228 (4.47), 292 (3.67)
IR ν_{max} (MeOH), cm^{-1} ; Figure 76	: 3377, 2932, 1672, 1598, 1458, 751
EIMS *m/z* (% relative intensity); Figure 77	: 338 (M^+,7), 323 (18), 307 (14), 306 (30), 295 (10), 277 (100), 263 (25), 223 (11), 214 (23), 213 (69)
^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)	: Figures 78-79, Table 20
^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)	: Figure 80, Table 20

5.9 Compound SVC-3

Compound SVC-3 was obtained as amorphous powder, soluble in chloroform.

Melting point	: 188°
UV λ_{max} (MeOH), nm (log ε); Figure 82	: 211 (3.72), 307 (3.09)
IR ν_{max} (MeOH), cm^{-1} ; Figure 83	: 3354, 2925, 2855, 1713, 1656, 1456, 1382, 754
ESMSMS *m/z* (positive ion mode); Figure 84	: 326 $(M+2H)^+$

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^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)	: Figures 85-86, Table 21
^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)	: Figure 87, Table 21

5.10 Compound SVC-4

Compound **SVC-4** was obtained as colorless amorphous powder, soluble in methanol.

UV λ_{max} (MeOH), nm (log ε); Figure 89	: 204 (6.74), 252 (6.32), 286 (5.98)
IR ν_{max} (KBr), cm^{-1} ; Figure 90	: 3372, 1698, 1461, 1419, 1372, 1239, 1202
EIMS *m/z* (% relative intensity); Figure 91	: 198 (M^+,100), 183 (31), 127 (12), 109 (22)
^{1}**H-NMR** (δ ppm, 300 MHz, CD_3OD)	: Figure 92, Table 22
^{13}**C-NMR** (δ ppm, 75 MHz, CD_3OD)	: Figure 93, Table 22

5.11 Compound SVC-5

Compound **SVC-5** was obtained as amorphous powder, soluble in methanol.

Melting point	: 209-212°
UV λ_{max} (MeOH), nm (log ε); Figure 94	: 210 (6.62), 263 (6.10)
IR ν_{max} (KBr), cm^{-1} ; Figure 95	: 3484, 3098, 2857, 1681, 1282, 1206
EIMS *m/z* (% relative intensity); Figure 96	: 168 (M^+, 100), 153 (77), 125 (22), 97 (22)
^{1}**H-NMR** (δ ppm, 300 MHz, CD_3OD)	: Figure 97, Table 23
^{13}**C-NMR** (δ ppm, 75 MHz, CD_3OD)	: Figure 98, Table 23

5.12 Compound SVC-6

Compound **SVC-6** was obtained as amorphous powder, soluble in methanol.

Melting point	: 126-127°
UV λ_{max} (MeOH), nm (log ε); Figure 99	: 206 (4.32), 277 (3.58)

IR ν_{max} (MeOH), cm^{-1}; Figure 100	: 3369, 2928, 1613, 1516, 1462, 1336, 1221, 1115
ESMSMS *m/z* (positive ion mode); Figure 101	: 965 $[M+Na]^+$
^{1}H-NMR (δ ppm, 300 MHz, CD_3OD)	: Figure 102, Table 24
^{13}C-NMR (δ ppm, 75 MHz, CD_3OD)	: Figure 103, Table 24

5.13 Compound SVC-7

Compound **SVC-7** was obtained as amorphous powder, soluble in methanol.

Melting point	: 126-127°
UV λ_{max} (MeOH), nm (log ε); Figure 113	: 208 (4.15), 277 (3.46)
IR ν_{max} (MeOH), cm^{-1}; Figure 114	: 3365, 2941, 1613, 1516, 1462, 1334, 1223, 1115, 1029
ESMSMS *m/z* (positive ion mode); Figure 115	: 765 $[M+Na]^+$
^{1}H-NMR (δ ppm, 300 MHz, CD_3OD)	: Figure 116, Table 25
^{13}C-NMR (δ ppm, 75 MHz, CD_3OD)	: Figure 117-118, Table 25

5.14 Compound SVH-1

Compound **SVH-1** was obtained as colorless needles, soluble in chloroform.

IR ν_{max} (KBr), cm^{-1} ; Figure 127	: 3500-3200, 3000-2800, 1640, 1470
^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)	: Figure 128
^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)	: Figures 129-130, Table 26

5.15 Compound SVH-2

Compound **SVH-2** was obtained as viscous oil, soluble in chloroform and methanol.

UV λ_{max} (MeOH), nm (log ε); Figure 131	: 242 (3.56)
IR ν_{max} (MeOH), cm^{-1}; Figure 132	: 3442, 3082, 2930, 2868, 1725, 1655, 1460, 1244, 1056
EIMS *m/z* (% relative intensity); Figure 133	: 302 (M^+, 6), 286 (22), 285 (100), 257 (9)
^{1}H-NMR (δ ppm, 300 MHz, CD_3OD)	: Figure 134-135, Table 27
^{13}C-NMR (δ ppm, 75 MHz, CD_3OD)	: Figure 136-137, Table 27

5.16 Compound SVH-3

Compound **SVH-3** was obtained as a orange needles, soluble in chloroform.

Melting point	: 198-200°
UV λ_{max} (MeOH), nm (log ε); Figure 146	: 224 (3.41), 302 (3.27)
IR ν_{max} (MeOH), cm^{-1}; Figure 147	: 3209, 2924, 2853, 1639, 1584, 1556, 1512, 1460, 1081, 739
EIMS *m/z* (% relative intensity); Figure 148	: 200 (M^+, 17), 171 (67), 143 (100), 115 (57)
^{1}H-NMR (δ ppm, 300 MHz, $CDCl_3$)	: Figure 149, Table 28
^{13}C-NMR (δ ppm, 75 MHz, $CDCl_3$)	: Figure 150, Table 28

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6. Determination of biological activities

6. 1 Antimicrobial activity

The activity was determined by microdilution method (Jorgensen *et al.*, 1999).

6.1.1 Microorganisms and media

Gram-negative *Escherichia coli* (ATCC 25922), gram-positive *Staphylococcus aureus* (ATCC 29213) and *Candida albicans* (ATCC 10231) were obtained from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. *E. coli* and *S. aureus* were cultured in Mueller-Hinton broth, whereas *C. albicans* was cultured in RPMI 1640.

6.1.2 Bioassay

The microorganisms were streaked on agar petri dish for isolation. The plates were incubated in a humidified incubator at 37 °C for 24 h, during which colonies were formed. With a sterile loop, the colonies were picked, dispersed in broth and incubated in a humidified incubator at 37 °C for another 24 h. After incubation, the concentration of the microorganisms was adjusted by diluting the overnight culture to a turbidity equal to that of 0.5 McFarland standard (80% transmittance at 625 nm). This standardized suspension has been shown to contain approximately 10^8 CFU/ml. The cell concentration was adjusted to 10^6 (bacteria) and 10^3 (yeasts) cells per 1 ml for use as the inoculum. Compound solution was added to the wells of a 96-well plate and serially diluted twofold. The final concentration of compound ranged from 1.56 to 800 μg/ml. After inoculation (50 μl/well for bacteria,100 μl/ well for yeasts, and none for control wells), the 96-well plate was incubated at 37 °C for 18 h. Then, 20 μl of 0.5 mg/ml *p*-iodonitrotetrazolium violet (INT) solution was added into each well. The microdilution plates were further incubated for 1 h for bacteria and overnight for *C. albicans*. The violet color of formazan developed indicates the microbial growth. The lowest concentration of isolated compounds that inhibited the

visible growth of test microorganism was regarded as the minimal inhibition concentration (MIC). Tetracycline and nystatin were used as reference agents.

6.2 Microplate assay for acetylcholinesterase inhibitory activity

Acetylcholinesterase inhibitory activity is determined by microplate assay (Ingkaninan *et al.*, 2000). The substrate ATCI (125 μl of 3 mM DTNB, 25 μl of 15 mM ATCI, and 50 μl of buffer) was mixed with 25 μl of sample dissolved in buffer. The microplate was then read by Bio-Rad microplate reader model 3550 UV (Bio-Rad Laboratories, Richmond, CA, USA) at 405 nm every 13 sec for 5 times. Then, 25 μl of 0.226 U/ml AchE solution was added to the wells and the microplate was read again at the same wavelength every 13 sec for 8 times. The increase of absorbance measured should be linear for more than 2 min. The velocities of the reaction before and after adding enzyme were calculated by a Microplate Manager software version 4.0 (Bio-Rad Laboratories). The results were corrected as a percentage compared to an assay using buffer without any inhibitor. Galanthamine was used as reference compound.

6.3 Cytotoxic activity

Assay for cytotoxic activity was performed as described by Leclerq *et al.* (1986). In each well of 96-well plates, 0.1 ml of medium containing 10,000 cells of melanoma B16 was inoculated and incubated in a humidified CO_2 incubator at 37°C. Twenty-four hours later, the medium was removed and replaced by 0.1 ml/well fresh medium containing various concentration of isolated compounds (maximum concentration 1000 μg/ml). After 72 h, the medium was eliminated and cell staining was performed with a 0.2% trypan blue solution in phosphate-buffered saline (PBS). The plates were then observed under an inverted phase contrast microscope. Trypan blue is used to determine cell membrane damage. Since the dye can enter into damaged cells, the dead cells will be stained while the viable cells will not.

6.4 Antioxidant activity

The reduction of 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) as described by Cavin *et al.* (1998) was performed. Ascorbic acid was used as reference compound.

TLC autographic assay: Thin layer chromatograms of the test compounds were developed. After drying, the plates were sprayed with 0.2 % DPPH solution in MeOH, then examined. Active compounds appear as yellow spots against purple background.

Spectrophotometric assay: The test compounds were mixed with 50 μl of 0.022% DPPH solution in MeOH and left at room temperature for 30 min. Absorbance at 550 nm was then determined and percentage of activity was calculated.