CHAPTER 2
EXPERIMENTAL

2.1 Instruments and Chemicals

Melting points were determined on the Fisher-John melting point apparatus. UV spectra were measured with a UV-160A spectrophotometer (Shimadzu) and principle bands (λ_{max}) were recorded as wavelengths (nm) and log ε in MeOH solution. The optical rotation $[\alpha]_D$ was measured in chloroform and methanol solution with Sodium D line (590 nm) on a JASCO P-1020 polarimeter. The IR spectra were measured with a Perkin-Elmer FTS FT-IR spectrophotometer and major bands (ν) were recorded in wave number (cm^{-1}). NMR spectra were recorded using 300 MHz Bruker FTNMR Ultra Shield^{TM} spectrometers. Chemical shifts were recorded in parts per million (δ) in deuterochloroform, deuteromethanol and deuteroacetone with tetramethylsilane (TMS) as an internal reference (δ 0.00 ppm). The EI-MS were performed using a MAT 95 XL mass spectrometer. Solvents for extraction and chromatography were distilled at their boiling point ranges prior to use except chloroform was analytical grade reagent. Quick column chromatography was carried out on silica gel 60H (Merck). Column chromatography was performed on silica gel (Merck) type 100 (0.063-0.200 nm). Precoated plates of silica gel 60 F_{254} (Merck) were used for analytical purposes. Sephadex LH-20 was also used for purification.

2.2 Plant Material

H. littoralis was collected from Songkhla province, Thailand in November 2004 and identified by Prof. Puangpen Sirirugsa, Department of Biology, Faculty of Science, Prince of Songkla University and specimen (No. CD01) was deposited at Prince of Songkla University Herbarium.

2.3 Extraction and Isolation

The air-dried bark of *H. littoralis* (6.0 kg) was extracted with hexane (2 x 30 L), CH_2Cl_2 (2 x 25 L) and acetone (2 x 25 L) successively for 5 days each at room temperature. The mixture was filtered and concentrated under reduced pressure to give crude extract of hexane (30.7 g), dichloromethane (32.0 g) and acetone (44.0 g), respectively. The process of extraction was shown in **Figure 2.**

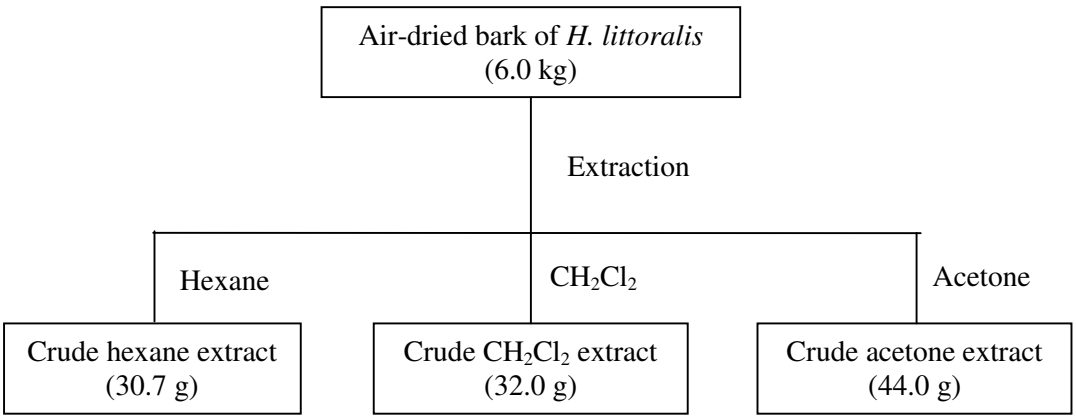
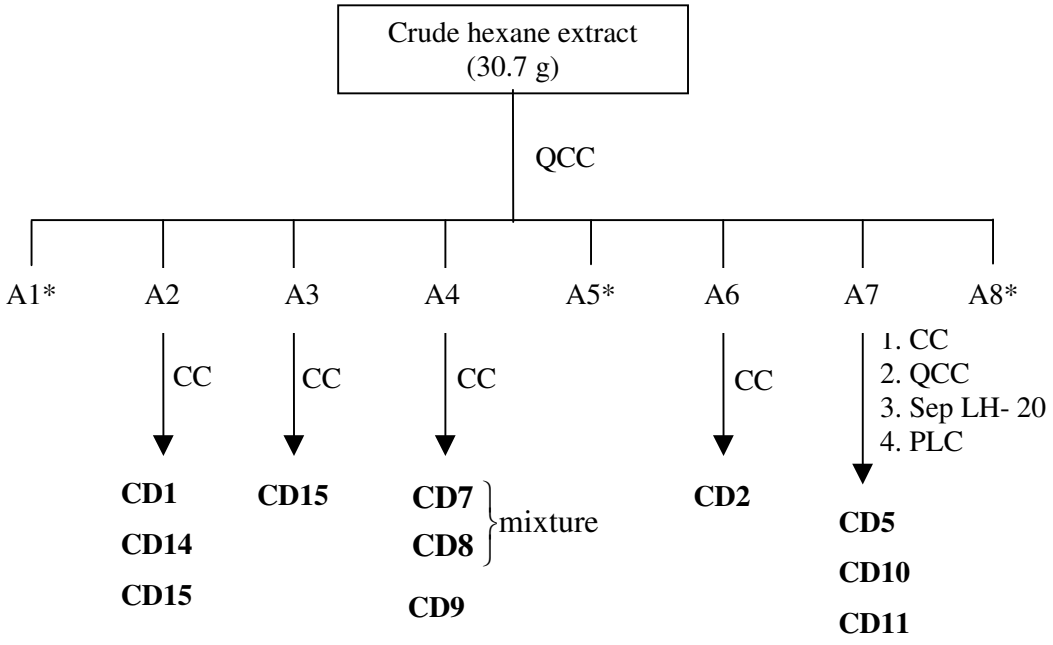


Figure 2 Extraction of the bark of *H. littoralis*

2.4 Isolation and Chemical Investigation

2.4.1 Investigation of the crude hexane extract from the bark of H. littoralis



*Not further investigated

Figure 3 Isolation of compounds **CD1, CD2, CD5, CD7, CD8, CD9, CD10, CD11, CD14** and **CD15** from the crude hexane extract

The crude hexane extract as a yellow viscous residue (30.7 g) was purified by quick column chromatography using silica gel and eluted with hexane and increasing polarity with CH_2Cl_2 and MeOH. On the basis of their TLC characteristic, the collected fraction which contained the same major components were combined: fractions A1-A8 were obtained (**Figure 3**).

Fraction A2 was filtered and washed with hexane to give a white solid of **CD1** (936.6 mg) (R_f = 0.23, 50% dichloromethane in hexane) and the mother liquor as yellow viscous oil (A2/1a) (2.1 g) after evaporation of the solvent.

The mother liquor (A2/1a) was rechromatographed on column chromatography using hexane as eluent and increasing polarity with ethyl acetate to afford four subfractions (A1/2a-A1/2d).

Subfraction A2/2b (68.7 mg) was rechromatographed on column chromatography and eluted with 15% ethyl acetate in hexane to give three subfractions (A2/3a-A2/3d), from which subfraction A2/3b (orange solid) was compound **CD14** (4.5 mg, R_f = 0.46, 15% ethyl acetate in hexane).

Only subfraction A2/3c (41.8 mg) was rechromatographed on column chromatography and eluted with 15% ethyl acetate in hexane to give **CD14** (15.7 mg).

Subfraction A2/2d (441.4 mg) as a yellow viscous oil was purified by column chromatography using 10% ethyl acetate in hexane to yield **CD15** (2.0 mg) as pale yellow needle solid (R_f = 0.37, 30% ethyl acetate in hexane).

Fraction A3 (2.42 g) was subjected to column chromatography using 10% acetone in hexane as eluent to give two subfractions (A3/4a-A3/4b).

Only subfraction A3/4b (292.4 mg) was rechromatographed on column chromatography using 25% ethyl acetate in hexane as eluent to give **CD15** (18.2 mg).

Fraction A4 (5.88 g) was purified by column chromatography using 60% dichloromethane in hexane as eluent to afford four subfractions (A4/5a-A4/5d).

Subfraction A4/5b (803.6 mg) as orange viscous oil was rechromatographed on column chromatography using 10% ethyl acetate in hexane as eluent to give **CD9** (43.3 mg) as a colorless viscous oil (R_f = 0.75, 15% ethyl acetate in hexane).

Subfraction A4/5c (298.7 mg) was filtered and washed with hexane to yield the mixture of **CD7** and **CD8** (7.6 mg) as a white solid (R_f = 0.30, 10% acetone in hexane).

Fraction A6 (1.53 g) was filtered and washed with hexane to give a white solid (35.6 mg) which was further subjected to column chromatography using 90% dichloromethane in hexane as eluent to afford **CD2** (4.8 mg) as a white solid (R_f = 0.41, 15% acetone in hexane) and three subfractions (A6/6b-A6/6d).

Only subfraction A6/6c (21.1 mg) was rechromatographed on column chromatography and eluted with 95% dichloromethane in hexane to give a white solid of **CD2** (5.6 mg).

Fraction A7 (4.76 g) was separated by quick column chromatography using hexane as eluent and increasing polarity with ethyl acetate to afford four subfractions (A7/7a-A7/7d).

Subfraction A7/7a (134.3 mg) was rechromatographed on column chromatography and eluted with 30% ethyl acetate in hexane to yield **CD5** (4.6 mg) as a white solid (R_f = 0.41, 30% ethyl acetate in hexane).

Subfraction A7/7c (1.51 g) was rechromatographed on column chromatography and eluted with 1% methanol in dichloromethane to yield four subfractions (A7/8a-A7/8d).

Subfraction A7/8b (200.0 mg) was subjected to column chromatography and eluted with 15% ethyl acetate in dichloromethane to give three subfractions (A7/9a-A7/9c).

Only subfraction A7/9b (77.1 mg) was purified by sephadex LH-20 and eluted with methanol to afford **CD10** (1.6 mg, R_f = 0.30, 20% ethyl acetate in dichloromethane) as a colorless viscous oil, and three subfractions (A7/10a, A7/10c and A7/10d).

Subfraction A7/10c (20.7 mg) was purified by preparative thin layer chromatography using 15% ethyl acetate in dichloromethane to afford **CD10** (12.6 mg).

Subfraction A7/8c (223.3 mg) was further purified by column chromatography using 20% ethyl acetate in dichloromethane as eluent to give four subfractions (A7/11a-A7/11d).

Subfraction A7/11c (23.5 mg) was purified by preparative thin layer chromatography using 4% methanol in dichloromethane to yield **CD11** (9.4 mg) as a colorless viscous oil (R_f = 0.53, 4% methanol in dichloromethane).

Compound CD1: white solid; mp 245-247 ^oC; $[\alpha]_D^{28}$: - 22.3^o (c = 0.54, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}): 1715 (C=O stretching); ^1H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 2**.

Compound CD2: white solid; mp 254-256 ^{o}C; $[\alpha]_{D}^{28}$: - 28.4^{o} (c = 0.31, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}): 3413 (O-H stretching) and 1715 (C=O stretching); ^{1}H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 3**.

Compound CD5: white solid; mp 280-282^{o}C; $[\alpha]_{D}^{28}$: + 17.7^{o} (c = 0.03, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}): 3413 (O-H stretching) and 1686 (C=O stretching) and 1645 (C=C stretching); ^{1}H and ^{13}C NMR ($CDCl_3$+CD_3OD) (δ ppm) (300 MHz): see **Table 6**.

Compounds CD7 and CD8: white solid; IR (KBr) ν_{max} (cm^{-1}): 3425 (O-H stretching), 2938 (C-H stretching) and 1642 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz).

Compound CD9: colorless viscous oil; $[\alpha]_{D}^{28}$: + 66.4^{o} (c = 0.40, $CHCl_3$); UV (MeOH) λ_{max} (nm) (log ε): 241 (4.21); IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 1674 (C=O stretching) and 1616 (C=C stretching); ^{1}H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 8**.

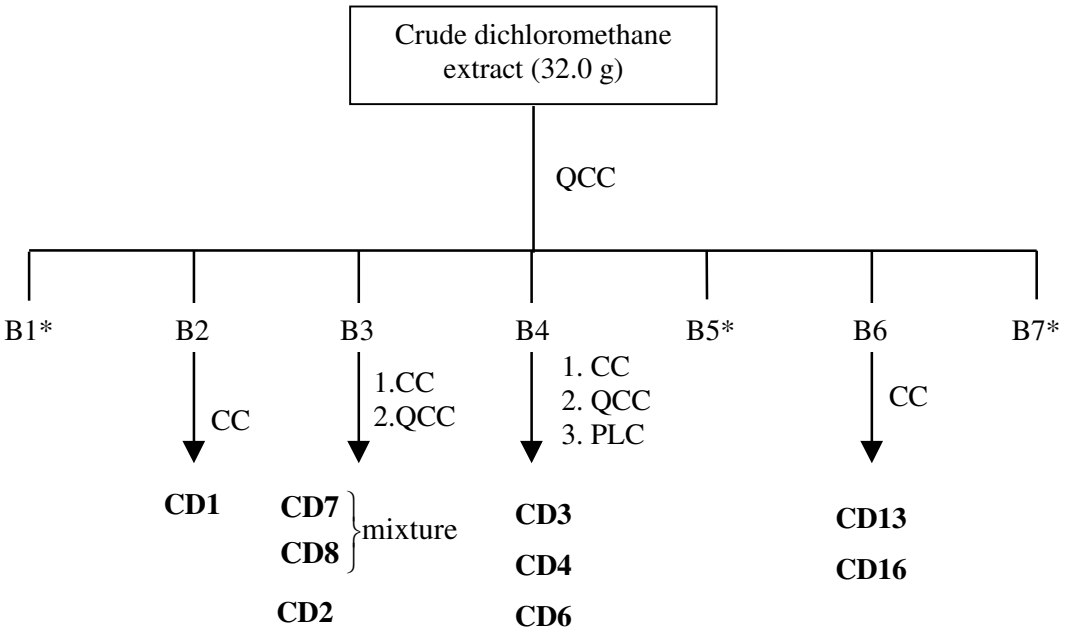
Compound CD10: colorless viscous oil; $[\alpha]_{D}^{28}$: + 10.7^{o} (c = 0.63, $CHCl_3$); UV (MeOH) λ_{max} (nm) (log ε): 237 (4.06); IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 3446 (O-H stretching), 1671 (C=O stretching) and 1642 (C=C stretching); EI-MS, [M^{+}] *m/z* 428.3653 (Calcd. for $C_{29}H_{48}O_2$: 428.6903); ^{1}H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 9**.

Compound CD11: colorless viscous oil; $[\alpha]_{D}^{28}$: + 16.0^{o} (c = 0.39, $CHCl_3$); UV (MeOH) λ_{max} (nm) (log ε): 241 (4.73) IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 3418 (O-H stretching), 1670 (C=O stretching) and 1645 (C=C stretching); EI-MS, [M^{+}] *m/z* 428.3668 (Calcd. for $C_{29}H_{48}O_2$: 428.6903); ^{1}H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 10**.

Compound CD14: orange solid; mp 208-209 ^{o}C; UV (MeOH) λ_{max} (nm) (log ε): 221 (3.39), 248 (3.14), 264 (3.11), 286 (3.08), 434 (2.85); IR (KBr) ν_{max} (cm^{-1}) : 3446 (O-H stretching), 1630 (C=O stretching), 1570 (C=C stretching); ^{1}H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 13**.

Compound CD15: pale yellow viscous oil; UV (MeOH) λ_{max} (nm) (log ε): 267 (4.19), 302 (3.63); IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 3393 (O-H stretching), 1625 (C=O stretching), 1441 (C=C stretching); ^{1}H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 14**.

2.4.2 Investigation of the crude dichloromethane extract from the bark of H. littoralis



*Not further investigated

Figure 4 Isolation of compounds **CD1, CD2, CD3, CD4, CD6, CD7, CD8, CD13** and **CD16** from the crude dichloromethane extract

The crude dichloromethane extract (32.0 g) as a dark green viscous oil was separated by quick column chromatography using silica gel as a stationary phase and eluted with hexane and increasing polarity with dichloromethane and methanol, successively to afford seven fractions B1-B7 (**Figure 4**).

Fraction B2 (1.48 g) was subjected to column chromatography using 15% acetone in hexane to give **CD1** (115.7 mg).

Fraction B3 (3.48 mg) was separated by quick column chromatography on silica gel with hexane and increasing polarity with ethyl acetate to give four subfractions (B3/1a-B3/1d).

Subfraction B3/1b was filtered and washed with hexane to afford **CD2** (14.0 mg).

Subfraction B3/1c was filtered and washed with hexane to give a mixture of **CD7** and **CD8** (40.6 mg).

Fraction B4 (2.40 g) was subjected to quick column chromatography and eluted with hexane and increasing polarity with ethyl acetate to give seven subfractions (B4/2a-B4/2g).

Subfraction B4/2b (675.7 mg) was filtered and washed with hexane to yield **CD3** (128.6 mg) as a white solid (R_f = 0.16, 95% dichloromethane in hexane).

Subfraction B4/2f (303.3 mg) was purified by column chromatography and eluted with 95% dichloromethane in hexane to afford four subfractions (B4/3a-B4/3d).

Only subfraction B4/3d (28.2 mg) was rechromatographed on column chromatography and eluted with 25% ethyl acetate in hexane to yield three subfractions (B4/4a-B4/4c).

Only subfraction B4/4b (13.6 mg) was purified by preparative thin layer chromatography using 30% ethyl acetate in hexane as eluent to give **CD6** (4.5 mg) as a pale yellow viscous oil (R_f = 0.26, 30% ethyl acetate in hexane).

Fraction B6 (785.7 mg) was separated by column chromatography using hexane and increasing polarity with ethyl acetate to afford three subfractions (B6/5a-B6/5c).

Only subfraction B6/5b was purified by column chromatography using 10% ethyl acetate in dichloromethane to give **CD13** (8.3 mg) as a colorless viscous

oil (R_f = 0.30, 15% ethyl acetate in dichloromethane) and **CD16** (14.4 mg) as a pale yellow viscous oil (R_f = 0.26, 15% ethyl acetate in dichloromethane).

Compound CD3: white solid; mp 256-257 ^oC; $[\alpha]_D^{28}$: - 121.7^o (c = 0.11, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}): 3493 (O-H stretching) and 1708 (C=O stretching); ^1H and ^{13}C NMR ($CDCl_3$ + CD_3OD) (δ ppm) (300 MHz): see **Table 4**.

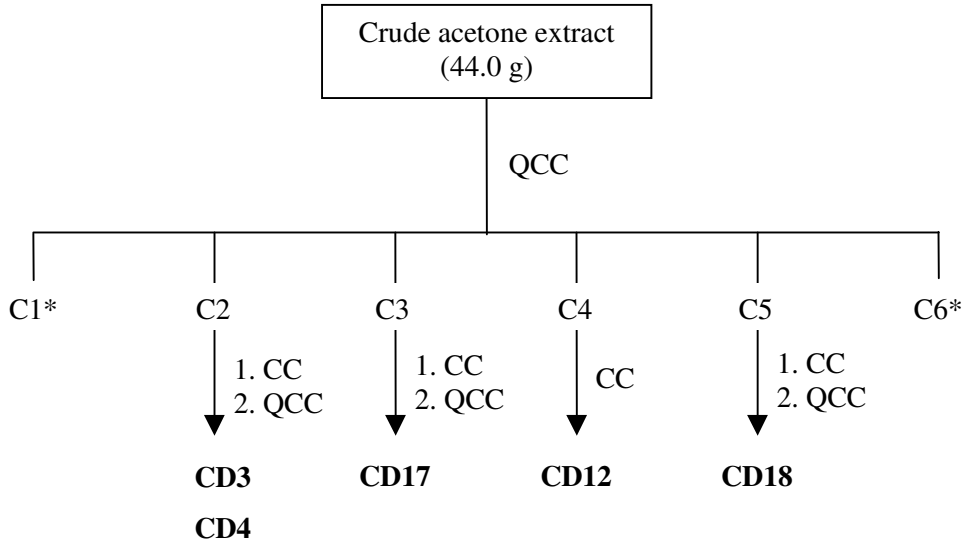
Compound CD4: white solid; mp 230-232 ^oC; $[\alpha]_D^{28}$: - 63.5^o (c = 0.22, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}): 3368 (O-H stretching) and 1705 (C=O stretching); ^1H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 5**.

Compound CD6: pale yellow viscous oil; $[\alpha]_D^{28}$: + 25.5^o (c = 0.23, $CHCl_3$); UV (MeOH) λ_{max} (nm) (log ε): 204 (4.03), 235 (3.85), 304 (3.89), 325 (4.02); IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 3375 (O-H stretching), 1695 (C=O stretching) and 1594 (C=C stretching); ^1H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 7**.

Compound CD13: colorless viscous oil; $[\alpha]_D^{28}$: - 12.8^o (c = 0.42, $CHCl_3$); IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 3442 (O-H stretching) and 1716 (C=O stretching); LREIMS: *m/z* (%) 428 $[M]^+$ (3.0), 410 $[M - H_2O]^+$ (5.5), 396 $[M - O_2]^+$ (100), 285 $[M - H_2O - (C_9H_{17})]^+$ (5.5), 253 $[M - O_2 - H_2O (C_9H_{17})]^+$ (15.0); ^1H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 12**.

Compound CD16: pale yellow viscous oil; $[\alpha]_D^{28}$: - 142.7^o (c = 0.72, $CHCl_3$); UV (MeOH) λ_{max} (nm) (log ε): 217 (3.76), 233 (3.82), 297 (3.89), 308 (3.90); IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 3432 (O-H stretching), 1744 (C=O stretching) and 1648 (C=C stretching); ^1H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 15**.

2.4.3 Investigation of the crude acetone extract from the bark of H. littoralis



*Not further investigated

Figure 5 Isolation of compounds **CD3, CD4, CD12, CD17** and **CD18** from the crude acetone extract

The crude acetone extract (44.0 g) as a dark red-brown viscous oil was purified by quick column chromatography using silica gel and eluted with gradient elution of hexane, dichloromethane and methanol. On the basis of their TLC characteristic, the collected fractions which contained the some major components were combined; fractions C1-C6 were obtained (**Figure 5**).

Fraction C2 (3.1 g) was separated by quick column chromatography using 70% dichloromethane in hexane as eluent to afford three subfractions (C2/1a-C2/1c).

Only subfraction C2/1b (606.5 mg) was purified by column chromatography using 20% ethyl acetate in hexane to give three subfractions (C2/2a-C2/2c).

Subfraction C2/2b was filtered and washed with hexane to afford **CD3** (33.3 mg).

Subfraction C2/2c was filtered and washed with hexane to yield **CD4** (6.6 mg).

Fraction C3 (6.4 g) was purified by quick column chromatography using 3% methanol in dichloromethane to give three subfractions (C3/3a-C3/3c).

Only subfraction C3/3b (572.1 mg) was subjected to quick column chromatography using 20% acetone in dichloromethane as eluent to give three subfractions (C3/4a-C3/4c).

Only subfraction C3/4b (47.7 mg) was rechromatographed on column chromatography using 30% ethyl acetate in hexane as eluent to afford **CD17** (9.4 mg) as a pale yellow viscous oil (R_f = 0.41, 30% ethyl acetate in dichloromethane).

Fraction C4 (1.2 g) was filtered and washed with hexane to give a white-brown solid. It was crystallized from 50% methanol in dichloromethane to afford **CD12** (31.5 mg) as a white solid (R_f = 0.21, 30% methanol in dichloromethane).

Fraction C5 (4.4 g) was purified by quick column chromatography using hexane as eluent and increasing polarity with acetone to afford four subfractions (C5/5a-C5/5d).

Only subfraction C5/5c (1.4 g) was rechromatographed on quick column chromatography and eluted with 10% methanol in dichloromethane to yield three subfractions (C5/6a-C5/6c).

Only subfraction C5/6b (745.9 mg) was further purified by quick column chromatography using 50% acetone in hexane as eluent to give three subfractions (C5/7a-C5/7c).

Subfraction C5/7b (323.6 mg) was crystallized with 80% methanol in dichloromethane to afford **CD18** (30.0 mg) as a pale yellow solid (R_f = 0.32, 60% acetone in hexane).

Compound CD12: white solid; mp 290-292 ^oC; $[\alpha]_D^{28}$: - 47.5^o (c = 0.10, MeOH); IR (KBr) ν_{max} (cm^{-1}): 3414 (O-H stretching), 1686 (C=O stretching); ^1H and ^{13}C NMR ($CDCl_3$+$CDCl_3$) (δ ppm) (300 MHz): see **Table 11**.

Compound CD17: pale yellow viscous oil; UV (MeOH) λ_{max}(nm) (log ε) : 206 (4.06), 227 (3.65), 278 (2.99); IR (neat $CHCl_3$) ν_{max} (cm^{-1}): 3335 (O-H stretching), 1600 (C=C stretching); ^1H and ^{13}C NMR ($CDCl_3$) (δ ppm) (300 MHz): see **Table 16**.

Compound CD18: pale yellow solid; mp 240-243 ^oC; $[\alpha]_D^{28}$: - 25.1^o (c = 0.23, MeOH); UV (MeOH) λ_{max} (nm) (log ε): 208 (4.70), 226 (4.29), 280 (3.64); IR (KBr) ν_{max} (cm^{-1}): 3453 (O-H stretching), 1623 (C=C stretching) and 1384 (C-O stretching); low resolution EI-MS, [M^+] *m/z* 290 (31); ^1H and ^{13}C NMR (acetone-d_6) (δ ppm) (300 MHz): see **Table 17**.

2.5 Anti-allergic activity assays

2.5.1 Inhibitory effect on the release of β-hexosaminidase from RBL-2H3 cells

Inhibitory effect on the release of β-hexosaminidase from RBL-2H3 were evaluated by the following method (Matsuda et al., 2002). Briefly, RBL-2H3 cells were dispensed in 24-well plates at a concentration of 2×10^5 cells/well using Minimum Essential Medium Eagle (MEM) containing 10% fetal calf serum (FCS), penicillin (100 units/mL), streptomycin (100 units/mL) and anti-DNP IgE (0.45 μg/mL), then incubated overnight at 37°C in 5% CO_2 for sensitization of the cells. The cells were washed twice with 500 μL of Siraganian buffer [119 mM NaCl, 5 mM KCl, 5.6 mM glucose, 0.4 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM piperazine-*N*,*N*′-bis (2-ethanesulfonic acid) (PIPES), 0.1 % BSA and 40 mM NaOH, pH 7.2] and then incubated in 160 μL of Siraganian buffer for an additional 10 min at 37 °C. After that, 20 μL of test sample solution were added to each well and incubated for 10 min, followed by addition of 20 μL of antigen (DNP-BSA, final concentration 10 μg/mL) at 37°C for 20 min to stimulate the cells to degranulate. The supernatant was transferred into 96-well plate and incubated with 50 μL of substrate (1mM *p*-

nitrophenyl-*N*-acetyl-β-D-glucosaminide) in 0.1 M citrate buffer (pH 4.5) at 37°C for 1 hr. The reaction was stopped by the addition of 200 μL of stop solution (0.1 M Na_2CO_3/$NaHCO_3$, pH 10.0). Measurement of absorbance was performed with a microplate reader at 405 nm. The test sample was dissolved in dimethylsulfoxide (DMSO), and the solution added to Siraganian buffer (final DMSO concentration 0.1 %). The inhibition (%) of the release of β-hexosaminidase by the test samples was calculated by the following equation, and IC_{50} values were determined graphically:

$$\text{Inhibition } \% = [1 - (T - B - N)/(C - N)] \times 100$$

Control (C): DNP-BSA (+), Test sample (–); Test (T) : DNP-BSA (+), Test sample (+); Blank (B) : DNP-BSA (–), Test sample (+); Normal (N) : DNP-BSA (–), Test sample (–)

2.5.2 β-Hexosaminidase inhibitory activity

In order to clarify that the anti-allergic effects of samples were due to the inhibition on β-hexosaminidase release, but not from the inhibition of β-hexosaminidase activity. The following assay was then carried out.

The cell suspension (5×10^7 cells) in 6 mL of PBS was sonicated. The solution was then centrifuged; and the supernatant diluted with Siraganian buffer and adjusted to equalize the enzyme activity of the degranulation tested above. The enzyme solution (45 μL) and test sample solution (5 μL) were transferred into a 96-well microplate and incubated with 50 μL of the substrate solution at 37°C for 1 hr. The reaction was stopped by adding 200 μL of the stop solution. The absorbance was measured using a microplate reader at 405 nm.

2.5.3 Statistics

The results were expressed as mean ± S.E.M of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. The statistical significance was calculated by one-way analysis of variance (ANOVA), followed by Dunnett's test.