CHAPTER 2
EXPERIMENTAL

2.1 Instruments and Chemicals

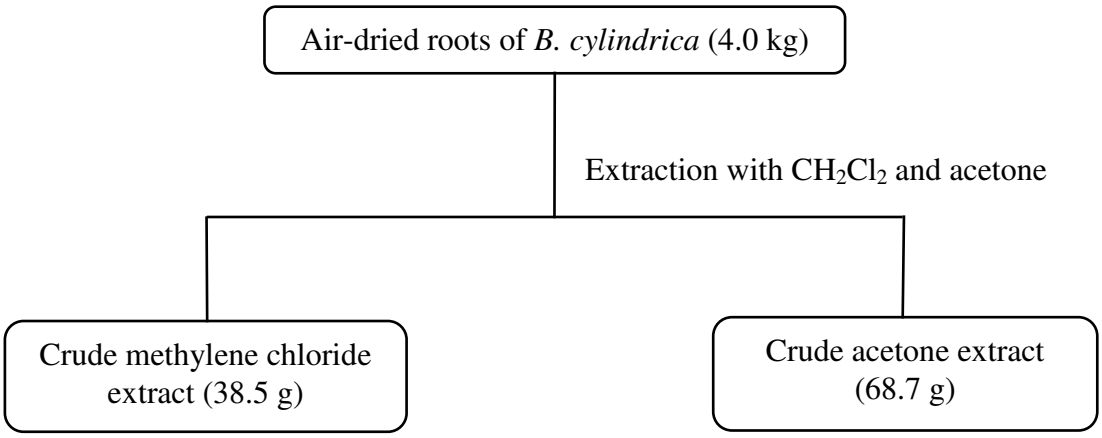
Melting points were determined on the Fisher-John melting point apparatus. UV spectra were measured with a UV-160A spectrophotometer (Shimadzu) and principle bands (λ_{max}) were recorded as wavelengths (nm) and log ε in MeOH solution. The optical rotation $[\alpha]_D$ was measured in chloroform and methanol solution with Sodium D line (590 nm) on a JASCO P-1020 digital polarimeter. The IR spectra were measured with a Perkin-Elmer FTS FT-IR and Shimudzu FTIR-8900 IR spectrophotometer. Single Crystal X-ray diffraction measurements were collected using SMART 1-K CDD diffractometer with monochromated Mo-Kα radiation (λ = 0.71073 A) using ω-scan mode and SHELXTL for structure solution and refinement. NMR spectra were recorded using 300, 400 and 500 MHz Bruker FTNMR Ultra Shield^{TM} spectrometers in $CDCl_3$ with TMS as the internal standard. Chemical shifts are reported in δ (ppm) and coupling constants (*J*) are expressed in hertz. EI and HREI mass spectra were measured on a Kratos MS 25 RFA 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 (QCC) and column chromatography (CC) were carried out on silica gel 60H (Merck) and silica gel 100 (Merck), respectively.

2.2 Plant Material

The roots of *B. cylindrica* were collected in December 2005 from Satun Province in the southern part of Thailand. This plant was identified by Prof. Puangpen Sirirugsa (voucher specimen no. 0012531). The voucher specimens have been deposited in the Herbarium of Department of Biology, Faculty of Science, Prince of Songkla University.

2.3 Extraction and Isolation

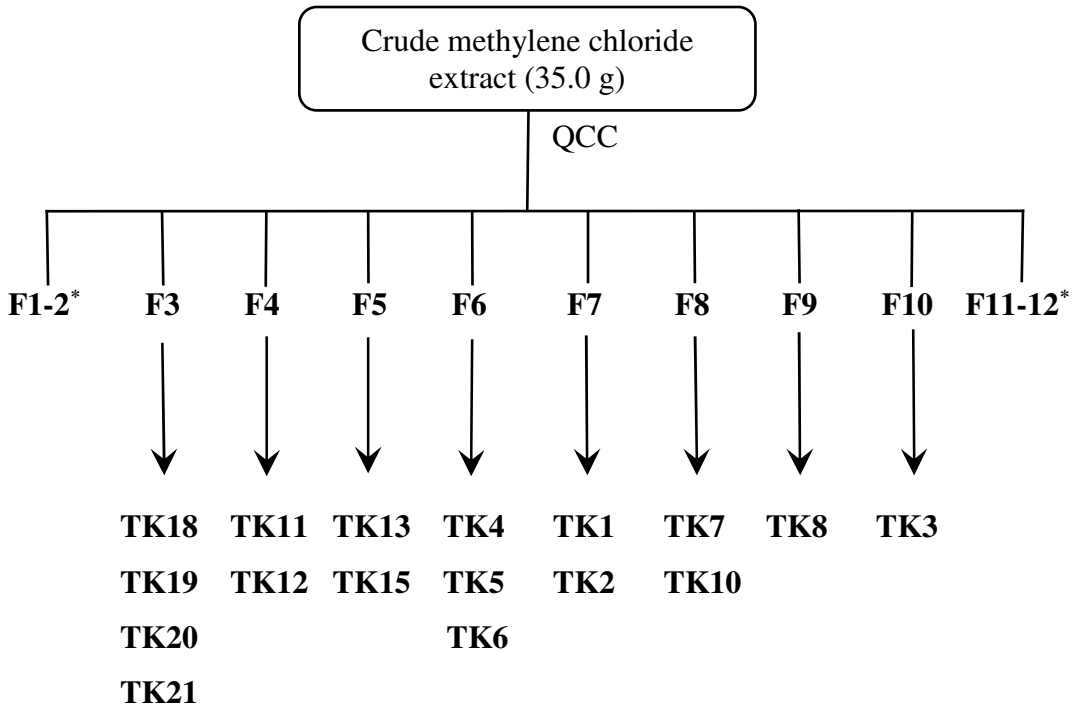
The air-dried and pulverized roots (4.0 kg) were exhaustively extracted with methylene chloride and acetone successively (2 x 22 L for each solvent for one week) at room temperature. The mixture was filtered and concentrated under reduced pressure to give crude extracts of methylene chloride (38.5 g) and acetone (68.7 g), respectively. The process of extraction was shown in **Scheme 1**.



Scheme 1 Extraction of the roots of *B. cylindrica*

2.4 Isolation and Chemical Investigation

2.4.1 Investigation of the crude methylene chloride extract from the roots of *B. cylindrica*



* No further investigation

Scheme 2 Isolation of compounds **TK1**-**TK5**, **TK7**-**TK13**, **TK15** and **TK18**-**TK21** from the methylene chloride extract.

A portion of the crude methylene chloride extract as a yellow viscous residue (35.0 g) was subjected to quick column chromatography over silica gel using solvent of increasing polarity from hexane through ethyl acetate. The eluates were collected and combined based on TLC characteristic to give twelve fractions (F1-F12).

Fraction F3 was filtered and washed with hexane to yield a mixture of **TK18**: β-sitosterol and **TK19**: stigmasterol (0.164 g) as a white solid and the mother liquor as yellow viscous oil (F3b) after evaporation of the solvent. The mother liquor

(F3b) was subjected to quick column chromatography using ethyl acetate-hexane (8:2) as eluting solvent to afford **TK20**: stigmast-4-en-3-one (37.4 mg) and three subfractions (F3b1-F3b3).

Only subfraction F3b3 (34.5 mg) was purified by preparative thin layer chromatography using hexane-acetone (6:4) to afford **TK21**: Erythrinassinate A (8.4 mg).

Fraction F4 (1.20 g) was subjected to quick column chromatography using hexane-acetone (9:1) as eluting solvent to afford **TK11**: lupeol (86.0 mg) and three subfractions (F4a-F4c). Only subfraction F4b (3.45 g) was rechromatographed on quick column chromatography using ethyl acetate-hexane (7:3) to yield **TK12**: lupenone (16.7 mg).

Fraction F5 (1.50 g) was separated by quick column chromatography using hexane-acetone (8:2) as eluting solvent to afford four subfractions (F5a-F5d).

Subfraction F5a (583.0 mg) was rechromatographed on quick column chromatography using hexane-ethyl acetate (7:3) as eluting solvent to afford **TK13**: betulinic acid (128.5 mg).

Subfraction F5b (234.0 mg) was rechromatographed on quick column chromatography using hexane-ethyl acetate (9:1) as eluting solvent to afford **TK15**: betulonic acid (79.5 mg).

Fraction F6 (2.80 g) was subjected to quick column chromatography using methylene chloride-acetone (8:2) as eluting solvent to afford **TK6**: *ent*-kaur-16-en-13-hydroxy-19-oic acid (173.1 g) and three subfractions (F6a-F6c).

Subfraction F6b (645.0 mg) was filtered and washed with hexane to give a white solid and followed by recrystallization from ethyl acetate-hexane (1:8) to yield **TK4**: *ent*-kaur-16-en-13-hydroxy-19-al (158.3 mg).

Subfraction F6b (1.67 g) was rechromatographed on quick column chromatography using methylene chloride-acetone (9:1) to give white solid and followed by recrystallization from hexane to yield **TK5**: *ent*-kaurenal (11.8 mg).

Fraction F7 (642.0 mg) was separated by quick column chromatography using methylene chloride-methanol (9.5:0.5) as eluting solvent to afford three subfractions (F7a-F7c).

Subfraction F7b (186.2 mg) was rechromatographed on quick column chromatography using methylene chloride-acetone (9:1) as eluting solvent to afford **TK2**: *ent*-kaurenol (7.6 mg).

Subfraction F7c (305.8 mg) was rechromatographed on quick column chromatography using methylene chloride-ethyl acetate (8:2) to give white solid and followed by recrystallization from acetone to yield **TK1**: *ent*-kaur-16-ene-13,19-diol (15.2 mg).

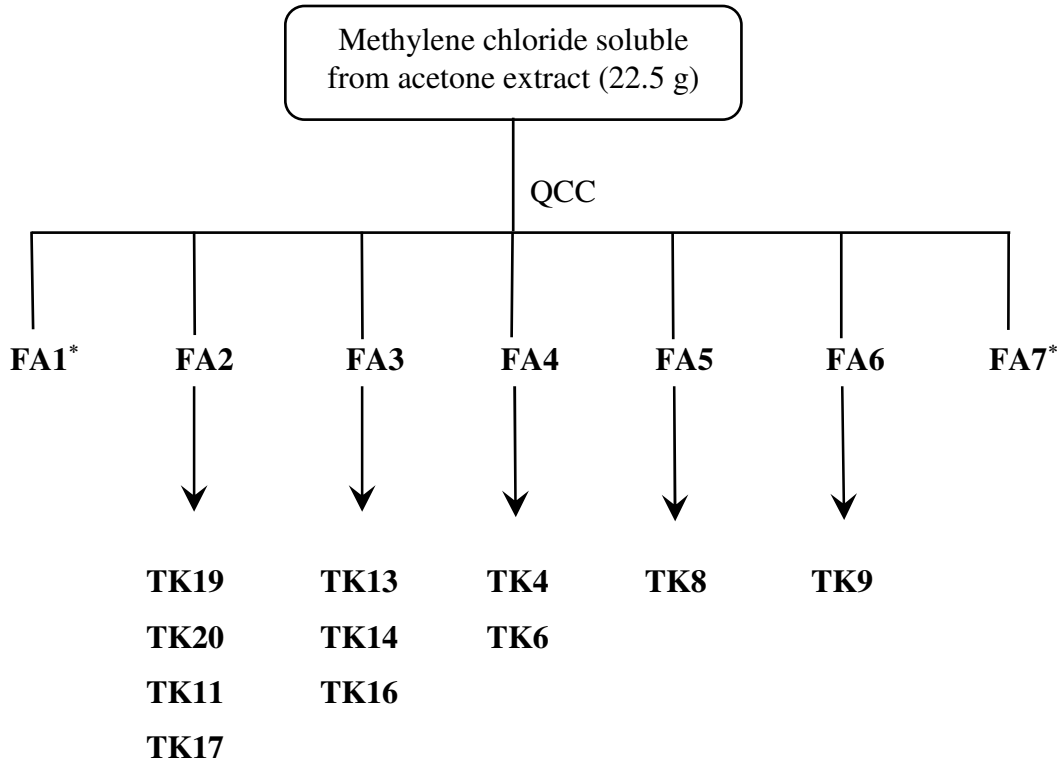
Fraction F8 (540.1 mg) was separated by quick column chromatography using hexane-acetone (7:3) as eluting solvent to afford **TK7**: methyl *ent*-kaur-9(11)-ene-13,17-epoxy-16-hydroxy-19-oate (21.0 mg) and four subfractions (F8a-F8d).

Subfraction F8b (265.7 mg) was rechromatographed on quick column chromatography using hexane-ethyl acetate (6:4) as eluting solvent to afford **TK10**: *ent*-8(9)-pimaren-15-one (33.2 mg).

Fraction F9 (920.2 mg) was subjected to quick column chromatography using methylene chloride-methanol (8:2) to give white solid and followed by recrystallization from acetone to yield **TK8**: *ent*-8,15*R*-epoxypimaran-16-ol (147.2 g).

Fraction F10 (1.10 g) was purified by quick column chromatography using methylene chloride-methanol (9:1) as eluting solvent to afford **TK3**: 16α*H*-17,19-*ent*-kauranediol (6.4 mg).

2.4.2 Investigation of the crude acetone extract from the roots of *B. cylindrica*



* No further investigation

Scheme 3 Isolation of compounds **TK4**, **TK6**, **TK8**, **TK11**, **TK13**, **TK14**, **TK16**, **TK17**, **TK19** and **TK20** from the acetone extract.

A portion of gummy residue from the acetone extract (50.0 g) was treated with methylene chloride to give methylene chloride-soluble and -insoluble fractions. The methylene chloride-soluble fraction was concentrated to afford gummy residue (22.5 g) which was subjected to quick column chromatography and eluted with hexane and ethyl acetate. The eluates were combined on the basis of TLC characteristic to give seven fractions (FA1-FA7).

Fraction FA2 (3.76 g) was separated by quick column chromatography using hexane-acetone (8:2) as eluting solvent to afford four subfractions (FA2a-

FA2d). Subfraction FA1a (583.6 mg) was filtered and washed with hexane to yield the mixture of **TK18**: β-sitosterol and **TK19**: stigmasterol (56.4 mg).

Subfraction FA2b (343.8 mg) was rechromatographed on quick column chromatography using hexane-ethyl acetate (9:1) as eluting solvent to afford **TK11**: lupeol (37.1 mg).

Subfraction FA2d (1.96 g) was rechromatographed on quick column chromatography using hexane-ethyl acetate (5:5) as eluting solvent to afford **TK17**: 30-nor-lupan-3β-ol-20-one (17.7 mg).

Fraction FA3 (380.1 mg) was subjected to quick column chromatography using hexane-acetone (7:3) as eluting solvent to afford **TK13**: betulinic acid (27.8 mg) and two subfractions (FA3a-FA3b).

Only subfraction FA3b (202.4 mg) was rechromatographed on quick column chromatography using hexane-ethyl acetate (6:4) as eluting solvent to afford **TK14**: 3-*epi*-betulinic acid (11.8 mg) and **TK16**: lup-20(29)-en-3β, 30-diol (16.7 mg).

Fraction FA4 (928.0 mg) was purified by quick column chromatography using methylene chloride-methanol (8:2) as eluting solvent to afford **TK4**: *ent*-kaur-16-en-13-hydroxy-19-al (25.0 mg) and **TK6**: *ent*-kaur-16-en-13-hydroxy-19-oic acid (41.6 mg).

Fraction FA5 (720.2 mg) was purified by quick column chromatography using methylene chloride-methanol (9.5:0.5) as eluting solvent to afford **TK8** : *ent*-8,15*R*-epoxypimaran-16-ol (67.4 mg).

Fraction FA6 (251.6 mg) was purified by quick column chromatography using methylene chloride-methanol (8.5:1.5) as eluting solvent to afford **TK9**: *ent*-17-hydroxy-16-ketobeyeran-19-oic acid (18.7 mg).

*Compound **TK1***, *ent*-kaur-16-en-13,19-diol: white amorphous solid; mp 255-257°C; $[\alpha]^{27}_{D}$ -22.7° (c = 0.30, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3292 (O-H stretching) and 1620 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (400 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (100 MHz): see **Table 2**.

Compound ***TK2***, *ent*-kaurenol: white amorphous solid; mp 140-141°C; $[\alpha]^{27}_{D}$ -75.0° (*c* = 0.34, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3350 (O-H stretching) and 1655 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 3**.

Compound ***TK3***, 16α*H*-17,19-*ent*-kauranediol: white amorphous solid, mp: 112-114°C; $[\alpha]^{27}_{D}$ -32.0° (*c* = 0.40, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3446 (O-H stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (400 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (100 MHz): see **Table 4**.

Compound ***TK4***, *ent*-kaur-16-en-13-hydroxy-19-al: white amorphous solid; mp 118-119°C; $[\alpha]^{27}_{D}$ -56.9° (*c* = 1.00, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3340 (O-H stretching) and 1712 (C=O stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 5**.

Compound ***TK5***, *ent*-kaurenal: white needles; mp 114-115°C; $[\alpha]^{27}_{D}$ -76.0° (*c* = 0.43, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 1718 (C=O stretching) and 1658 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 6**.

Compound ***TK6***, *ent*-kaur-16-en-13-hydroxy-19-oic acid: white amorphous solid; mp 199-201°C; $[\alpha]^{27}_{D}$ -58.1° (*c* = 2.00, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}): 3396 (O-H stretching) and 1687 (C=O stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 7**.

Compound ***TK7***, methyl *ent*-kaur-9(11)-ene-13,17-epoxy-16-hydroxy-19-oate: white amorphous solid, mp 169-171°C; $[\alpha]^{27}_{D}$ +36.3° (*c* = 0.40, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3427 (O-H stretching) and 1728 (C=O stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 8**.

Compound ***TK8***, *ent*-8,15*R*-epoxypimaran-16-ol: white amorphous solid, mp: 85-86°C; $[\alpha]^{27}_{D}$ -67.2° (*c* = 0.01, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3346 (O-H stretching); ^1H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 9**.

Compound ***TK9***, *ent*-17-hydroxy-16-ketobeyeran-19-oic acid: white amorphous solid, mp: 230-232°C; $[\alpha]^{27}_{D}$ -35.0° (*c* = 0.30, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 3535 (O-H stretching) and 1719, 1650 (C=O stretching); ^1H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 10**.

Compound ***TK10***: pale yellow viscous oil, $[\alpha]^{27}_{D}$ +53.2° (*c* = 0.50, $CHCl_3$); IR (KBr) ν_{max} (cm^{-1}) 1728 (C=O stretching) and 1649 (C=C stretching); ^1H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 11**; HREIMS *m/z* $[M]^+$ 288.2479 (Calcd for $C_{20}H_{32}O$, 288.2500).

Compound ***TK11***, lupeol: white solid, mp: 193-194 °C; $[\alpha]^{28}_{D}$: +25.0° (*c* = 0.20, $CHCl_3$); FT-IR (KBr) ν_{max} (cm^{-1}): 3343 (O-H stretching), 2945 (C-H stretching) and 1638 (C=C stretching); ^1H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 12**.

Compound ***TK12***, lupenone: white solid, mp: 163-165 °C; $[\alpha]^{28}_{D}$: +50.0° (*c* = 0.10, $CHCl_3$); FT-IR (KBr) ν_{max} (cm^{-1}): 2914 (C-H stretching), 1704 (C=O stretching) and 1642 (C=C stretching); ^1H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 13**.

Compound ***TK13***, betulinic acid: white solid, mp: 279-280 °C; $[\alpha]^{28}_{D}$: +15.0° (*c* = 0.10, $CHCl_3$); FT-IR (KBr) ν_{max} (cm^{-1}): 3415 (O-H stretching), 2942 (C-H stretching), 1686 (C=O stretching) and 1645 (C=C stretching); ^1H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 14**.

Compound ***TK14***, 3-*epi*-betulinic acid: white solid, mp: 257-259 °C; $[\alpha]^{28}_{D}$: -10.0° (*c* = 0.05, $CHCl_3$); FT-IR (KBr) ν_{max} (cm^{-1}): 3436 (O-H stretching), 2947 (C-H stretching), 1704 (C=O stretching) and 1643 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 15**.

Compound ***TK15***, betulonic acid: white solid, mp: 250-254 °C; $[\alpha]^{28}_{D}$: +32.0° (*c* = 0.37, MeOH); FT-IR (KBr) ν_{max} (cm^{-1}): 3326 (O-H stretching), 1704 (C=O stretching) and 1642 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 16**.

Compound ***TK16***, lup-20(29)-en-3β, 30-diol: white solid, mp: 203-204 °C; $[\alpha]^{28}_{D}$:-13.3° (*c* = 0.15, $CHCl_3$); FT-IR (KBr) ν_{max} (cm^{-1}): 3416 (O-H stretching), 2926 (C-H stretching) and 1635 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 17**.

Compound ***TK17***, 30-nor-lupan-3β-ol-20-one: white solid, mp: 234-235 °C; $[\alpha]^{28}_{D}$: -22.7° (*c* = 0.22, $CHCl_3$); FT-IR (KBr) ν_{max} (cm^{-1}): 3414 (O-H stretching), 2941 (C-H stretching), 1694 (C=O stretching) and 1643 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 18**.

Compound ***TK18,*** β-sitosterol and ***TK19***, stigmasterol: white solid, FT-IR (neat) ν_{max} (cm^{-1}): 3425 (O-H stretching) and 1642 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 20**.

Compound ***TK20***, stigmast-4-en-3-one: colorless viscous oil; $[\alpha]^{28}_{D}$: +66.4° (*c* = 0.40, $CHCl_3$); UV (MeOH) λ_{max} (nm) (log ε): 241 (4.21); FT-IR (neat) ν_{max} (cm^{-1}): 1674 (C=O stretching) and 1616 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 19**.

Compound ***TK21***, erythrinassinate A: colorless viscous oil, UV (MeOH) λ_{max} (nm) (log ε): 325 (4.04), 297 (3.96) and 234 nm (4.01); FT-IR (KBr) ν_{max} (cm^{-1}): 3375 (O-H stretching) 1695 (C=O stretching) and 1635 (C=C stretching); ^{1}H NMR ($CDCl_3$) (δ ppm) (300 MHz) and ^{13}C NMR ($CDCl_3$) (δ ppm) (75 MHz): see **Table 21**; EIMS *m/z* $[M]^+$ 586.6 (Calcd for $C_{38}H_{66}O_4$, 586.5).

2.5 Bioassays

2.5.1 Cytotoxic assay

The procedure for cytotoxic assay was performed by the sulphorhodamine B (SRB) assay as described by Skehan et al. (Skehan et al., 1990). In this study, three cancer cell lines obtained from National Cancer Institute, Bangkok, Thailand, were used: MCF-7 (breast adenocarcinoma), KB (human oral cancer) and HeLa (Human cervical cancer). Camptothecin which was used as a standard showed cytotoxic activity in the range of 0.2-2.0 μg/mL.