CHAPTER III

CHEMICAL CONSTITUENTS OF SILKWORM EXCRETA

3.1 Introduction

Silkworm excreta are abundant waste in sericulture. They have been known for the medicinal purposes in Eastern Asia for long time. Several research groups worked in isolation of chlorophylls and their derivatives from silkworm excreta because they have been reported for the therapeutic activity as mentioned in chapter II. By the way, there are groups of compounds in silkworm excreta which have not been investigated. Thus the isolation and structure elucidation of the compounds could lead to the discovery of useful chemical constituents, particularly, the compounds which possess the fascinating bioactivity.

3.2 Material and Methods

3.2.1 Source of the sample

Silkworm (*Bombyx mori* L.) strain Lueng Korat excreta were collected from Queen Sirikit Sericulture Center, Nakhonratchasima, Thailand in September 2008. Sample was air-dried in the shade by local farmers to protect from microbial deterioration before given. After received, visible foreign matters such as small pieces of plant leaves, dead silkworm bodies, etc. were sorted out by hands. Sample was dried at 60^{o}C for 5 hours. Otherwise, fresh sample was kept at -20^{o}C unless extraction was done within the day received.

Mulberry (*Morus alba* L., Burerum-60 cultivar) leaves which used in silkworm rearing were given as a plant specimen as well. A voucher specimen (ML 050951) has been deposited at Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.

3.2.2 General techniques

3.2.2.1 Analytical thin layer chromatography

Analytical thin layer chromatography (TLC) was used to study the pattern of crude silkworm excreta extracts and in checking and gathering the fractions obtained from column chromatography. One dimensional and ascending techniques were used for developing the Silica gel 60 GF_{254} (E. Merck) precoated plate (0.2 mm thickness). The distance between the origin to the solvent front was fixed at 6 cm. All experiments were done in laboratory temperature which was about 30-35°C. Observing the TLC plate under ultraviolet light at wavelengths of 254 and 365 nm and spraying of anisaldehyde with sulphuric acid followed by heating at 105°C for 10 min were used as detection method.

3.2.2.2 Column chromatography

3.2.2.2.1 Vacuum column chromatography

Silica gel 60 (No.7734) with particle size 0.063-0.200 nm (70-230 mesh ASTM) (E.Merck) was used as an absorbent. Sample was dissolved in a small amount of the initial eluent, mixed with a small quantity of adsorbent, triturated, dried and then put on top of the column by dry method. Fractions were examined by the same way as described in section 3.7.1 then grouped and given the fraction name by regarding to the similar TLC pattern.

3.2.2.2.2 Flash column chromatography

Silica gel 60 (No.9385) with particle size 0.040-0.063 nm (70-230 mesh ASTM) (E. Merck) was used as an absorbent. Sample was dissolved in a small amount of eluent and then applied gently on top of the silica gel which was already packed by wet method within the column. Fractions were examined by the same way as described in section 3.2.1 then grouped and given the fraction name by regarding to the similar TLC pattern.

3.2.2.2.3 Gel filtration chromatography

Gel filter (Sephadex LH 20, GE Healthcare) was suspended in the eluent and left standing to swell for 24 hours before using. After the gel filter was set tightly in the column, dissolved sample was applied gently on top of the column. Fractions were examined by the same way as described in section 3.2.1 then combined regarding to the similar TLC pattern.

3.2.2.2.4 High performance liquid chromatography (HPLC)

HPLC was applied in the characterization of the crude extracts obtained by extraction with different solvents. Zorbax C-18 ODS (0.5 mm i.d. x 250 mm) column was used. HPLC grade solvents were purchased from Lab Scan, Thailand. Mobile phase was acetronitrile : MeOH (3:1). The sample was dissolved in the eluent to get 1 mg/ml sample solutions. Then, sample solutions were filtered through Millipore nylon membrane filter (0.45 micron) before injection. Injection volume was 20 μL. System was run by LC-8A (Shimadzu) pump at the flow rate of 1.0 ml/min. The detector and recorder were SPD-10A UV Detector (Shimadzu) and C-R6A Chromatopac (Shimadzu). All experiments were done in room temperature.

3.2.2.3 Spectroscopy

3.2.2.3.1 UV-visible spectroscopy

UV/visible spectra of the crude extract (in methanol) were obtained on a Shimadzu UV-160A UV/visible spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

3.2.2.3.2 Proton and carbon-13 nuclear magnetic resonance (^{1}H and ^{13}C-NMR) spectroscopy

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

Deuterated chloroform ($CDCl_3$) was used as the solvent for NMR. Chemical shifts were recorded in ppm scale using the chemical shift of the solvent as the reference signal.

3.2.2.3.3 Mass spectrometry

Micromass LCT spectrtometer or a Thermo-Finnigan Polaris Q mass spectrometer (Department of Chemistry, Faculty of Science, Mahidol University) were used for observing the mass spectra.

3.2.2.3.4 Fourier transformed infrared spectroscopy (FT-IR)

Fourier transformed infrared spectrometer Spectral Perkin Elmer Spectral one (Scientific and Technological Research Equipment Center, Chulalongkorn University) was used to obtained FT-IR spectra.

3.2.2.4 Chemicals and solvents

Commercial grade organic solvents employed throughout this work were redistilled prior to use.

3.2.3 Extraction of silkworm excreta

Four solvents were selected for extraction. Hot water (80-100^{o}C) was selected due to the primitive procedure of making a medicinal tea from silkworm excreta. Ethanol was chosen because of low toxicity and edible. And, acetone as well as

hexane was picked from their ease to remove filter cake, reasonable price, and availability to adapt for the extraction in industrial scale.

Equal amount of dried sample was macerated separately in each solvent, in the proportion of 1:5 w/v, for 1 hour. Then, extraction was accelerated by sonication for 15 minutes. Extraction mixtures were filtered through Whatman No.1 filter paper with vacuum. The filtered cakes which had remaining color were washed by the extracting solvents until the filtrate becomes colorless. Filtrates from each solvent were pooled and evaporated until dry under reduced pressure in rotary evaporator. Temperature during evaporation was controlled to be lower than 35°C with protection of the extracts from direct light. Finally, crude extracts were obtained and kept in 4-8°C prior to the test of free radical scavenging and tyrosinase inhibition activity and then was subjected to the separation.

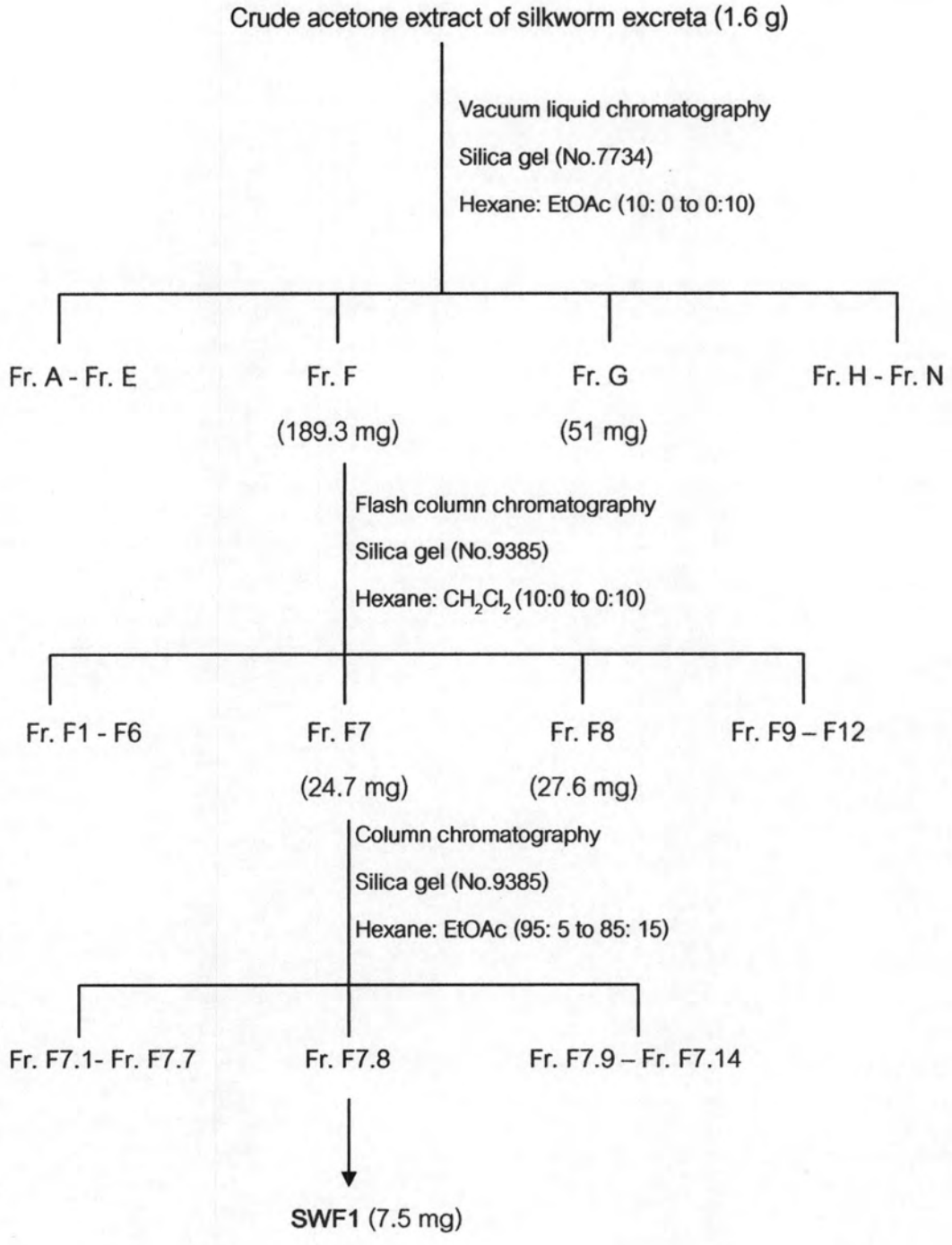
3.2.4 Isolation of chemical constituents from silkworm excreta

The crude acetone extract (1.6 g) was separated by vacuum liquid column chromatography using a sintered glass filter column of silica gel (No.7734, 112 g). The acetone extract was dissolved in a small amount of acetone, triturated with silica gel (No.7734) and dried under vacuum. Elution was performed in a polarity gradient manner with mixtures of hexane and ethyl acetate from 0 % ethyl acetate in hexane to 100% ethyl acetate. The fractions were collected 50 ml each to yield 81 fractions and examined by TLC (silica gel, hexane: ethyl acetate 4:1). Fractions with similar chromatographic manner were combined to yield 14 fractions: A (fraction 1-10), 90.0 mg; B (fraction 11-21), 109.9 mg; C (fraction 22-25), 116 mg; D (fraction 26-34), 142.7 mg; E (fraction 35-39), 133.1 mg; F (fraction 40-43), 189.3 mg; G (fraction 44-46), 51 mg; H (fraction 47-50), 183.3 mg; I (fraction 51-54), 140.3 mg; J (fraction 55-58), 161.5 mg; K (fraction 59-63), 61.7 mg; L (fraction 64-65), 31.4 mg; M (fraction 66-70), 52.7 mg; N (fraction 71-80), 115.2 mg.

3.2.4.1 Isolation of compound SWF1

The isolation of SWF1 was illustrated in Scheme 1. Fraction F (189.3 mg) was fractionated on a silica gel (No. 9385) column. Elution was performed in a polarity gradient manner with mixtures of hexane and dichloromethane (from 0% dichloromethane in hexane to 100% dichloromethane). Fractions (83 fractions) showing similar chromatographic pattern were combined (TLC, silica gel, hexane: ethyl acetate, 4:1; hexane: dichloromethane, 1:5 and 3:5) to yield 12 fractions: F1 (8.4 mg), F2 (11.4 mg), F3 (10.6 mg), F4 (34.5 mg), F5 (21.2 mg), F6 (22.8 mg), F7 (24.7 mg), F8 (27.6 mg), F9 (13.8 mg), F10 (5.4 mg), F11 (2.8 mg), F12 (3.5 mg)

F7 (24.7 mg) was purified on a silica gel (No. 9385) column. Elution was performed in a polarity gradient manner with mixtures of hexane and ethyl acetate (from 5% to 15% ethyl acetate in hexane) to give F7.8 (7.5 mg, 10.84×10^{-3}% based on dried weight of excreta) appeared as white solid (SWF1). The compound was identified as 1-tritriacontanol.



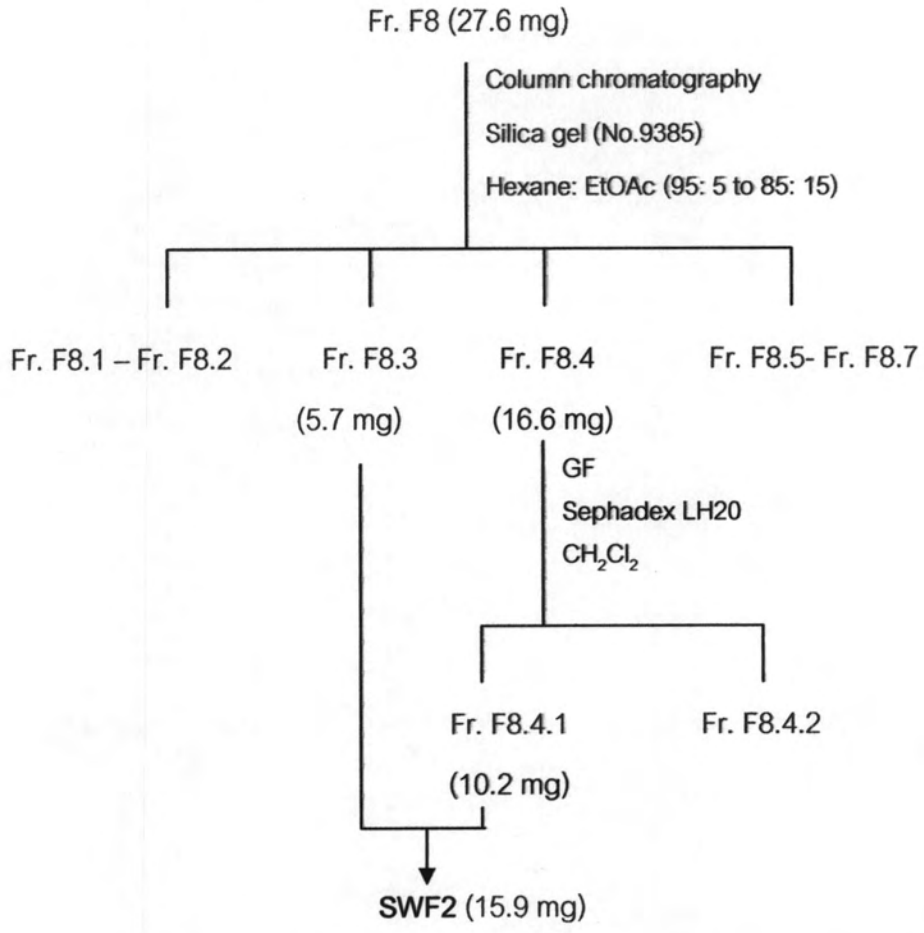
Scheme 1 Isolation of SWF1 (1-tritriacontanol) from crude acetone extract

3.2.4.2 Isolation of compound SWF2

Fraction F8 (27.6 mg) was purified on a silica gel (No. 9385) column. Elution was performed in a polarity gradient manner with mixtures of hexane and ethyl acetate (from 5% to 15% ethyl acetate in hexane) to give 7 fractions F8.1 (1.1 mg), F8.2 (0.9 mg), F8.3 (5.7 mg), F8.4 (16.6 mg), F 8.5 (0.9 mg), F8.6 (0.4 mg), and F8.7 (1.3 mg)

F8.3 (5.7 mg) showed one purple spot on TLC with anisaldehyde spray (R_f 0.4, silica gel, hexane: ethyl acetate 4:1). After dry, white needles were found.

F8.4 (16.6 mg) has 2 overlapped purple spots on TLC with anisaldehyde spray (R_f 0.38 and 0.4, silica gel, hexane: ethyl acetate 4:1). Thus, underwent further purification on Sephadex LH20 column (dichloromethane) and pooled with F8.3 to give compound SWF2 as white needles (15.9 mg, R_f 0.4, silica gel, hexane: ethyl acetate 4:1). Eventually, SWF2 (15.9 mg, 23.06×10^{-3}% based on dried weight of excreta) was identified as lupeol.



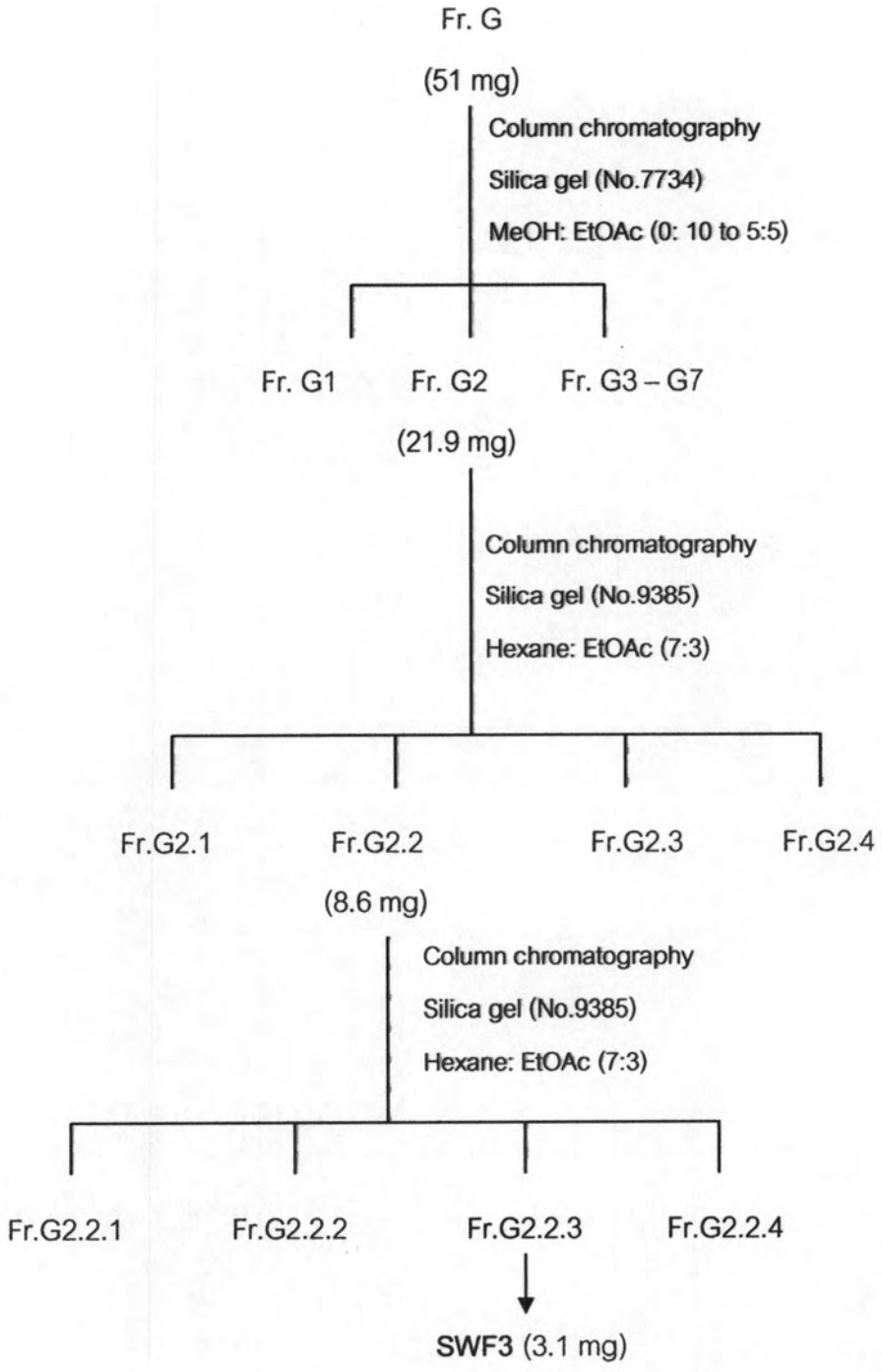
Scheme 2 Isolation of SWF2 (lupeol) from crude acetone extract

3.2.4.3 Isolation of compound SWF3

Purification of G (51 mg) was performed on a silica gel (No. 9385) column. The gradient elution was methanol in ethyl acetate (0 to 50% methanol in ethyl acetate). The eluates (31 fractions) were combined based on their TLC patterns (silica gel, hexane: ethylacetate, 8:1, 7:3, and 1:1) to yield 7 fractions: G1 (0.8 mg), G2 (21.9 mg), G3 (6.7 mg), G4 (3.4 mg), G5 (1.3 mg), G6 (12.5 mg), G7 (0.8 mg)

G2 (21.9 mg) was further separated on a silica gel (No. 9385) column. Isocratic elution of 30% ethyl acetate in hexane was applied. Twenty-seven fractions were obtained and were combined based on their TLC patterns to yield 5 fractions (G2.1-G2.4). White needles were found in fraction G2.2 (8.6 mg).

G2.2 (8.6 mg) was purification with silica gel (No. 9385) column using isocratic elution of 10% ethyl acetate in hexane. White needles (SWF3) were found in fraction G2.2.3 (3.1 mg). Single spot (R_f 0.22) was detected by anisaldehyde spray on the TLC (silica gel, hexane: ethylacetate, 10:1, twice developed). SWF3 (3.1mg, 4.50 $x10^{-3}$% based on dried weight of excreta) was identified as β-sitosterol.



Scheme 3 Isolation of SWF3 (β-sitosterol) from crude acetone extract

3.2.5 Physical and spectral data of isolated compounds

3.2.5.1 Compound SWF1 (1-tritriacontanol)

Compound SWF1 was obtained as white solid, soluble in CH_2Cl_2 (7.5 mg, $10.84x10^{-3}$% based on dried weight of excreta)

^{1}H-NMR : δ ppm, 300 MHz, in $CDCl_3$; Appendix Figure A, Table 3

^{13}C-NMR : δ ppm, 75 MHz, in $CDCl_3$; Appendix Figure B, Table 4

IR : ν_{max} cm^{-1}, KBr disc; Appendix Figure C

3412 (br), 2918, 2849, 1473, 1463, 1379, 1059

EIMS : *m/z* (% relative intensity); Appendix Figure D

480.17 (M^+, 2), 426.18 (6), 411.25 (9), 259.21 (14), 218.18 (100), 203.20 (25), 189.19 (33), 175.17 (13), 111.14 (20), 97.16 (25)

3.2.5.2 Compound SWF2 (lupeol)

Isolated SWF2 was obtained as colorless needles, soluble in CH_2Cl_2 (15.9 mg, $23.06x10^{-3}$% based on dried weight of excreta)

^{1}H-NMR : δ ppm, 300 MHz, in $CDCl_3$: Appendix Figure E, Table 5

^{13}C-NMR : δ ppm, 75 MHz, in $CDCl_3$: Appendix Figure F and G, Table 6

EIMS : *m/z* (% relative intensity); Appendix Figure H

426.14 (M^+, 12), 412.25 (12), 333.19 (30), 287.18 (19), 241.26 (27), 218.25 (34), 207.07 (43.61), 190.23 (53), 189 (100), 175.27 (37), 159.11 (24), 149.25 (31), 147.19 (58), 121.18 (34), 109.20 (38), 95.19 (55), 81.03 (49), 67.08 (43)

3.2.5.3 Compound SWF3 (β-sitosterol)

Isolated SWF3 was obtained as colorless needles, soluble in CH_2Cl_2 (3.1mg, $4.50x10^{-3}$% based on dried weight of excreta)

^{1}H-NMR : δ ppm, 300 MHz, in $CDCl_3$: Appendix Figure I and J, Table 7

^{13}C-NMR : δ ppm, 75 MHz, in $CDCl_3$: Appendix Figure K, L, M, and N, Table 8

EIMS : *m/z* (% relative intensity); Appendix Figure O

414.23 (M^+, 44), 396.31 (32), 381.27 (21), 329.16 (59), 303.26 (18.94), 273.19 (38), 255.27 (62), 231.17 (37), 213.16 (81), 189.19 (33), 173.23 (37), 171.22 (44), 163.22 (50), 160.20 (60), 145.13 (82), 131.18 (61), 107.15 (56), 105.13 (100), 91.15 (81), 79.07 (62), 67.03 (37)

IR : ν_{max} cm^{-1}, KBr disc; Appendix Figure P

3434 (br), 2936, 2958, 2869, 1634, 1465, 1382, 1053

3.2.6 Bioactivity test of chemical compounds

3.2.6.1 Free radical scavenging activity

The stable free radical, 1,1-diphenyl-2-picrylhydrazyl (DPPH) were used to measured antioxidant activity of the crude extracts. The assays based on a single electron transfer reaction, monitor through a change in color as the oxidant is reduced (Huang, Ou, and Prior, 2005: 1841-1856).

3.2.6.1.1 TLC screening assay (Ayoola *et al.*, 2008)

Samples were dissolved in methanol and spotted on Silica gel 60 F_{254} (E. Merck) precoated plate (0.2 mm thickness). The plates were developed in appropriate solvent system for each sample. Then, 0.2% w/v solution of DPPH in methanol was sprayed on the TLC plates. The plates were visualized for the presence of yellowish spots on the purple background.

3.2.6.1.2 Determination of free radical scavenging activity

A sample was diluted with methanol to make the test solution at the concentration of 20-800 μg/mL. One hundred micromolar DPPH solution was prepared

by dissolving 2 mg of DPPH in 100 ml of methanol, and stir for 30 min to make DPPH completely soluble. The DPPH solution was then kept into sealed bottle. Protect the solution from direct light by wrapping the bottle with aluminium foil. Free radical scavenging activity was assessed by the previously described method (Choi *et al.*, 2008, Zheng *et al.*, 2008) with slightly modification. Each of test samples (20 μL) was added into 180 μL DPPH solution (100 μM) in the 96-well plate. The reaction mixture was incubated at room temperature in the dark, for 30 min and then the absorbance of each well was measured at 510 nm by the microplate reader (Multilabel counter 102, Perkin Elmer, USA). Sample blanks for each crude extract were prepared due to an interference of the color of sample at measuring wavelength by replacing DPPH solution with an equal volume of methanol. Quercetin was used as a reference compound. The negative control was consisted of methanol (20 μL) and DPPH solution (180 μL). The free radical scavenging activity was calculated by equation 1.

$$\text{DPPH scavenging activity (\%)} = [A_c-(A_s-A_{sb})]/A_c \times 100 \qquad (1)$$

Where A_c is the absorbance of the control sample

A_s is the absorbance of the test sample

A_{sb} is the absorbance of the sample blank.

3.2.6.2 Tyrosinase inhibitory activity

Tyrosinase inhibition activity was determined by spectrophotometry, as described previously by Lim (Lim *et al.*, 2009) with minor modifications. Isolated compounds were diluted with methanol to make the 20-800 μg/mL test sample. Mushroom tyrosinase was freshly prepared by dissolving 0.15 mg of mushroom tyrosinase (100 Units) in 100 ml of 0.1 M phosphate buffer (pH 6.8). The enzyme was freezed unless testing was done within the same day. Sample (40 μL) was added to 80

μL of 0.1 M phosphate buffer (pH 6.8). Then, added 40 μL of mushroom tyrosinase (0.15 mg/mL). After 10 min, 40 μL of L-DOPA (0.85 mM) was added. Then, the reaction mixtures were incubated for 30 min, the absorbance of the resultant DOPA chromophore was measured at 490 nm using microplate reader (multi-label counter 102, Perkin Elmer, USA). Each sample was accompanied by blank that had almost the same component except the substrate, L-DOPA. Results were compared with a control which was replaced the sample by methanol. Kojic acid was used as a positive control. The percent inhibition of tyrosinase activity was calculated by equation 2.

$$\text{Tyrosinase inhibition (\%)} = [(A_c - A_s)/A_c] \times 100 \qquad (2)$$

Where A_c is the absorbance of the control sample

A_s is the absorbance of the test sample

3.3 Result and discussion

3.3.1 Isolation of chemical compounds

3.3.1.1 Isolation of SWF1

SWF1 was a white solid which negative to anisaldehyde spray. The compound was identified as tritriacontanol by the ^{1}H, ^{13}C NMR, and Mass spectral data.

The ^{1}H NMR spectrum of SWF1 as in Appendix, Figure A and Table 3 showed signals at δ 3.66 (2H, *t*, *J* = 6.6 Hz) due to H-1 which attach to hydroxyl bonded carbon C-1. The signal at δ 1.58 (2H, *bs*) was due to H-2. The signal at δ 1.27 (60H, *m*) was 30 methylene units (H-3 to H-32). The signal at δ 0.87 (3H, *t*, *J* = 6.9 Hz) was of the terminal methyl group.

The ^{13}C NMR spectrum (Figure B, Table 4) show signal at δ 63.11 due to oxygenated carbon. A group of CH_2 around δ 29 suggested the linear long chain structure. Other carbon positions were assigned in Table 4.

IR spectrum (Figure C) showed a broad band at 3412 cm^{-1} due to the hydroxyl group. Peaks at 2849 and 2920 cm^{-1} was due to C-H stretching.

Mass Spectrum (Figure D) showed molecular ion at *m/z* 480, chemical formula $C_{33}H_{68}O$ was predicted.

Comparison of these data with the previous report (Rao *et al.*, 2009), the compound SWF1 was identifed as tritriacontanol.

$$HO\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}(CH_2)_{27}\text{-}CH_2\text{-}CH_2\text{-}CH_3$$

1 2 3 4-30 31 32 33

Figure3 tritriacontanol

Table 3 ^{1}H-NMR spectral data for SWF1

Carbon position	δ (multiplicity, *J* (Hz))
1	3.66 (2H, *t*, *J* = 6.6 Hz)
2	1.58 (2H, *bs*, *J* = 6.3 Hz)
3-32	1.27 (60H, *m*)
33	0.87 (3H, *t*, *J* = 6.9 Hz)

Table 4 Proposed carbon assignment for SWF1

Carbon position	Chemical shift (ppm)
1	63.12
2	32.82
3	25.74
4	29.42
5	29.65
6	29.56
7	29.35
8	22.68
9	14.09

3.3.1.2 Isolation of SWF2

SWF 2 was isolated as white needles which showed purple spot on anisaldehyde detection. The compound was finally identifed as lupeol by comparison to the previous data of ^{1}H and ^{13}C NMR.

The ^{1}H NMR spectral data (Figure E and Table 5) of SWF2 showed down field signals at δ 4.70 and 4.59 (each 1H, *s*) due to the olefinic protons (H-29). The signal at δ 3.22 and 3.19 (1H, *dd*, *J*= 5.4, 6.6 Hz) represented H-3 which is the proton at the hydroxyl attached carbon. The signal at δ 1.70 was of the methyl proton which substituted at C-30. The signals at δ 1.05, 0.99, 0.96, 0.85, 0.81, and 0.78 were the methyl proton which substituted at C-23, C-24, C-25, C-26, C-27, and C-28.

The ^{13}C NMR spectrum (Figure F, G and Table 6) exhibited 30 signals of terpenoid lupine skeleton. The carbon bonded to hydroxyl group (C-3) appeared at δ 78.8. The olefinic carbon signals were downfield to δ 150.7 and 109.3 (C-20 and C-29). Seven methyl singlets were found. Comparison of these data with ^{13}C NMR data of lupeol (Imam *et al.*, 2007) is shown in Table 6.

The mass spectrum (Figure H) showed molecular ion at *m/z* 426 (12%), the molecular formula $C_{30}H_{50}O$ was predicted.

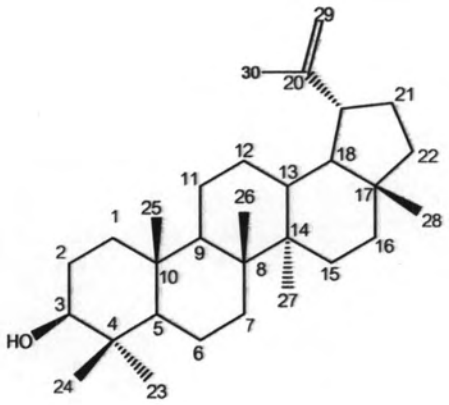
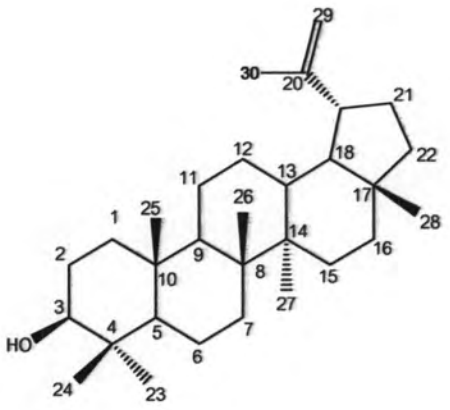


Figure 4 lupeol

Table 5 ^{1}H-NMR spectral data of SWF2 and lupeol

^{1}H-NMR signal of SWF2 ($CDCl_3$)	^{1}H-NMR signal of lupeol ($CDCl_3$)	Proton position
4.70, 4.59 (each 1H, *bs*)	4.69, 4.56 (each 1H, *m*)	H-29
3.22, 3.19 (1H, *dd*, *J*= 5.4, 6.6 Hz)	3.20 (1H, *m*)	H-3
2.38 (1H, *m*)	2.39 (1H, *m*)	H-19
1.70 (3H, *s*)	1.69 (3H, *s*)	H-30
1.05, 0.99, 0.96, 0.85, 0.81, and 0.78 (each 3H, *s*)	1.04, 0.98, 0.97, 0.84, 0.82, and 0.79 (each 3H, *s*)	H-23 to H-28



lupeol

Table 6 ^{13}C-NMR spectral data of SWF2 and lupeol

Carbon position	Chemical shift (ppm)		Carbon position	Chemical shift (ppm)	
	SWF2	lupeol		SWF2	lupeol
1	38.7	38.6	16	35.6	35.5
2	27.4	27.3	17	43.0	42.9
3	79.0	78.9	18	48.3	48.2
4	38.7	38.8	19	48.0	47.9
5	55.3	55.2	20	150.7	150.8
6	18.3	18.3	21	29.9	29.8
7	34.3	34.2	22	40.0	39.9
8	40.8	40.7	23	28.0	27.9
9	50.5	50.3	24	15.4	15.3
10	37.1	37.1	25	16.1	16.1
11	21.0	20.9	26	16.0	15.9
12	25.2	25.0	27	14.6	14.5
13	38.1	38.0	28	18.0	18.0
14	42.8	42.7	29	109.3	109.3
15	27.5	27.4	30	19.3	19.2

3.3.1.3 Isolation of SWF3

SWF3 was isolated as colorless needles. When detect with anisaldehyde TS, the spot appeared as purple, indicated the steroidal or triterpenoid skeleton. It was identified as β-sitosterol. The SWF3 was compared with the authentic sample by TLC before confirm the structure by its ^{1}H and ^{13}C NMR data with previous reported values (Wright *et al.*, 1978).

The ^{1}H NMR spectrum (Figure I, J, and Table 7) of SWF3 showed signals at δ 0.67-1.03 ppm which were the signals of six methyl protons that substituted at side chain of the steroidal skeleton. The signal at δ 3.54 ppm (m) represented H-3 which is the proton at the hydroxyl attached carbon. The olefinic signal at δ 5.36 ppm (1H, *d*, *J*= 5.1 Hz) could be assigned as H-6. The data was compared to those of previous report (Hahnvajanawong *et al.*, 2005).

The ^{13}C NMR spectrum (Figure K, L, and Table 7) and the DEPT 90° and 135°spectrum (Figure M) showed 30 carbon signals including 3 quaternary, 9 methine, 11 methylene, and 6 methyl carbons. The olefinic carbon (C-5 and C-6) signals were found downfield at δ 140.77 and 121.71. The signal at δ 71.82 represented the C-3 which attached to hydroxyl group. Comparison of these data with ^{13}C NMR data of β-sitosterol (Wright *et al.*, 1978) is shown in Table 7.

The IR spectral (Figure O) showed O-H stretching peak at 3434 cm^{-1}. Peak at 2959, 2869 cm^{-1} was the stretching of C-H bond. C=C stretching peak was shown at 1634 cm^{-1}. C-H bending peak at 1465 and 1383 cm^{-1} were found.

The structure was confirmed by Mass spectrum (Figure P) which exhibited a molecular ion peak at *m/z* 414 (43.7%) corresponding to molecular formula $C_{29}H_{50}O$.

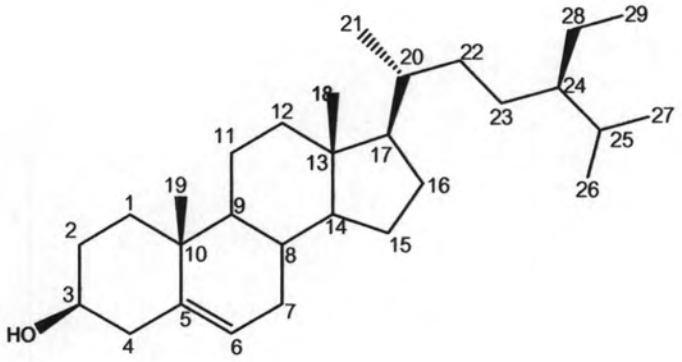
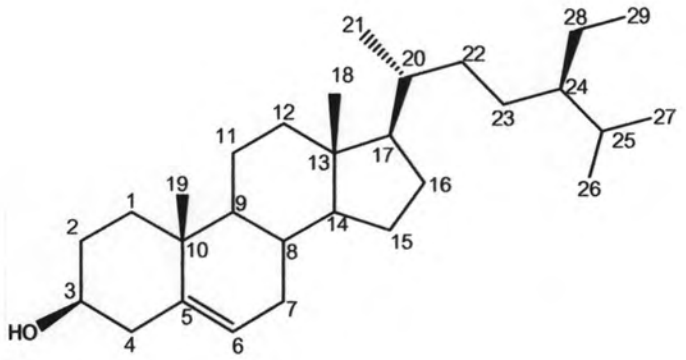


Figure 5 β-sitosterol

Table 7 ^{1}H-NMR spectral data of SWF3 and β-sitosterol

^{1}H-NMR signal of SWF3 ($CDCl_3$)	^{1}H-NMR signal of β-sitosterol ($CDCl_3$)	Proton
5.37 (1H, *d*, *J*= 5.1 Hz)	5.35 (1H, *d*, *J*= 4.3 Hz)	H-6
3.54 (1H, *m*)	3.50 (1H, *bs*)	OH
2.31-1.20 (30H, *m*)	2.30-1.10 (30H, *m*)	methine and methylene
1.05 (3H, *s*)	1.02 (3H, *s*)	H-19
0.97-0.79 (3Hx4, *m*)	0.99-0.78 (3Hx4, *m*)	H-21, H-26, H-27, and H29
0.69 (3H, *t*, *J*= 6 Hz)	0.7 (3H, *s*)	H-18



β-sitosterol

Table 8 ^{13}C- NMR Spectral data of SWF3 and β-sitosterol

Carbon position	Chemical shift (ppm)	
	SWF3	β-sitosterol
1	37.27	37.31
2	31.69	31.57
3	71.82	71.69
4	42.23	42.25
5	140.77	140.76
6	121.71	121.59
7	31.92	31.92
8	31.92	31.92
9	50.17	50.17
10	36.52	36.51
11	21.10	21.11
12	39.80	39.81
13	42.33	42.33
14	56.79	56.79
15	24.30	24.32

Carbon position	Chemical shift (ppm)	
	SWF3	β-sitosterol
16	28.24	28.26
17	56.00	56.11
18	11.86	11.87
19	19.39	19.40
20	36.15	36.17
21	18.78	18.82
22	33.98	33.95
23	26.15	26.13
24	45.88	45.85
25	11.86	12.32
26	19.80	19.84
27	19.04	19.07
28	23.10	23.09
29	11.99	12.32

3.3.2 Bioactivity of isolated compounds

3.3.2.1 Free radical scavenging activity of compound SWF1, SWF2, SWF3, and SWF4

For the test of free radical scavenging activity, isolated compounds were first test at 100 μg/ml. Evaluation for IC_{50} values was done for compounds that exhibited more than 50% inhibition. Quercetin was employed as positive control. The results are summarized in Table 9. Three pure compounds were tested for free radical scavenging activity. It was found that all of them showed low activity against DPPH free radical as compared with quercetin.

Table 9 Percentage of DPPH reduction by isolated compounds from silkworm excreta

Compounds	% DPPH reduction at 100 μg/ml	IC_{50} (μM)
SWF1	1.66	-
SWF2	5.07	-
SWF3	2.50	-
Quercetin	53.94	6.07

3.3.2.2 Tyrosinase inhibitory activity of compound SWF1, SWF2, SWF3, and SWF4

For the evaluation of tyrosinase inhibitory activity, isolated compounds were also test at 100 μg/ml. From the result in Table 10, it was found that all of isolated compounds had weak tyrosinase inhibitory activity (2.31, 10.04, and 5.23 % tyrosinase inhibition) as compared with the positive control, Kojic acid.

Table 10 Percentage of tyrosinase inhibition by isolated compounds from silkworm excreta extract

Compounds	% tyrosinase inhibition at 100 μg/ml
SWF1	2.31
SWF2	10.04
SWF3	5.23
Kojic acid	51.67

3.4 Conclusion

The isolation and structure elucidation of the chemical compounds from silkworm excreta was successfully done and 3 compounds were obtained.

SWF1 was identified as 1-tritriacontanol which is the fatty alcohol found in carnuba wax (Deroux *et al.*, 2003). This compound was isolated for the first time from silkworm excreta with the yield of 10.84×10^{-3}% based on dried weight of excreta.

SWF2 (23.06×10^{-3}% based on dried weight of excreta) and SWF3 (4.50×10^{-3}% based on dried weight of excreta) were identified as Lupeol and β-sitosterol, respectively. Both of them were found in mulberry leaves (Butt *et al.*, 2008). Thus finding these compounds in silkworm excreta indicates that lupeol and β-sitosterol from mulberry were maintained during digestion and excretion process of silkworm. Lupeol was reported for its anticancer activity (Saleem *et al.*, 2004, Saleem *et al.*, 2005, Saleem *et al.*, 2009) as well as anti-inflammatory activity (Geetha and Varalashmi, 2001) while β-sitosterol was reported for angiogenic activity (Choi *et al.*, 2002) anti inflammatory activity (Prieto *et al.*, 2006), and cholesterol reducing activity (Field *et al.*, 1997) and known as beneficial plant sterol.

Successful isolation of three active compound leads to expand the use of silkworm excreta as a new source of fatty alcohol, a potent anticancer and an advantageous phytosterol.