CHAPTER IV

RESULTS AND DISCUSSION

Data analysis of 6 fractions

LFD-H1; white amorphous solid; m.p. = 73.0 - 76.0°C; IR (ν_{max} KBr): cm^{-1} 2917.8, 2849.0, 1473.2, 1462.9, 1378.5; ^1H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^1H NMR profile of saturated hydrocarbon reported in the reference (Pouchert, et al., 1993).

LFD-H2; white amorphous solid; m.p. = 72.5 - 74.5°C; IR (ν_{max} KBr): cm^{-1} 2917.7, 2849.0, 1736.7, 1473.2, 1462.9, 1378.1, 1175.2; ^1H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^1H NMR profile of wax ester reported in the reference (Pouchert, et al., 1993).

LFD-H3; colorless needle; ^1H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^1H NMR profile reported in the literature (Muhit et al., 2010), corresponding to the structure of 17-(5-ethyl-6-methylheptan-2-yl)-10,13-dimethyl-2,3,4,7,8,9,11,12,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3 -ol (*β*-sitosterol). ^1H NMR (400 MHz, acetone-d_6) and ^{13}C NMR (100 MHz, acetone-d_6) spectral data were shown in table 2.

LFD-H4; white amorphous solid; IR (ν_{max} neat): cm^{-1} 2917.3, 2849.6, 1743.4, 1463.3, 1362.2, 1170.8; ^1H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^1H NMR profile of fatty acid methyl ester reported in the reference (Pouchert, et al., 1993).

LFD-D1; white amorphous solid; m.p. = 85.0 - 88.0°C; IR (ν_{max} neat): cm^{-1} 2917.4, 2849.4, 1472.7, 1463, 1735.3; ^1H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^1H NMR profile of fatty aldehyde reported in the reference (Pouchert, et al., 1993).

LFD-D2; white amorphous solid; m.p. = 80.1 - 83.0°C; IR (ν_{max} neat): cm^{-1} 3436.5, 2916.9, 2873.9, 1472.8, 1462.7, 1062.3; ^1H NMR (400 MHz, $CDCl_3$) data of

Fraction LFD-D2 was trimethylsilylated. It was labeled as **LFD-D2-Silyl**. Fractions LFD-H1, LFD-H2, LFD-H4, LFD-D1 and LFD-D2-Silyl were further investigated by GC-MS and the results were shown in table 3

Table 2 ^{13}C and ^{1}H NMR spectral data for LFD-H3 (β-sitosterol) and literatures

Atoms	LFD-H3 (β-sitosterol)		Literatures	
	δ_C	δ_H, multiplicity, *J* in Hz	δ_C[(a)]	δ_H, multiplicity, *J* in Hz[(b)]
1	37.41	-	37.33	-
2	28.85	-	31.63	-
3	71.98	3.51, *m*	71.73	3.51, *m*
4	42.46	-	42.00	-
5	140.92	-	140.71	-
6	121.88	5.34, *br s*	121.16	5.39, *m*
7	31.82	-	31.96	-
8	32.07	-	31.81	-
9	50.29	-	51.13	-
10	36.66	-	36.43	-
11	21.24	-	21.09	-
12	39.93	-	39.79	-
13	42.46	-	42.37	-
14	56.93	-	56.75	-
15	24.46	-	24.15	-
16	28.40	-	28.75	-
17	56.22	-	56.02	-
18	12.01	0.68, *s*	11.84	0.72, *s*
19	19.55	1.00, *s*	19.46	1.05, *s*
20	36.30	-	36.07	-
21	19.19	0.92, *d*, 6.5	18.68	0.96, *d*, 6.5
22	34.11	-	33.95	-
23	29.31	-	26.10	-
24	46.00	-	45.82	-
25	26.24	-	29.51	-
26	18.93	0.83, *d*, 6.5	19.77	0.87, *d*, 6.7
27	19.96	0.80, *d*, 6.6	19.21	0.85, *d*, 6.7
28	23.23	-	23.13	-
29	12.13	0.84, *t*, 7.2	11.04	0.89, *t*, 7.4

Note: [(a)] Sharma, et al., 2010 [(b)] Muhit, et al., 2010

Table 3 Chemical composition analysis of LFD-H1, LFD-H2, LFD-H4, LFD-D1 and LFD-D2-Silyl by GC-MS

Fractions	Compounds found	Molecular weight	Peak area %
LFD-H1	- Heptacosane ($C_{27}H_{56}$)	380	0.64
	- Nonacosane ($C_{29}H_{60}$)	408	6.10
	- Hentriacotane ($C_{31}H_{64}$)	436	86.41
LFD-H2	- Nonacosane ($C_{29}H_{60}$)	408	2.47
	- Hentriacotane ($C_{31}H_{64}$)	436	76.07
	- Tritriacotane ($C_{33}H_{68}$)	464	18.98
LFD-H4	- Hexadecanoic acid methyl ester (methyl palmitate) ($C_{17}H_{34}O_2$)	270	87.83
	- Octadecanoic acid methyl ester (methyl stearate) ($C_{19}H_{38}O_2$)	298	5.94
	- Eicosanoic acid methyl ester (methyl arachidate) ($C_{21}H_{42}O_2$)	326	1.04
LFD-D1	- Octadecanal ($C_{18}H_{36}O$)	268	42.52
	- Hentriacotane ($C_{31}H_{64}$)	436	52.04
LFD-D2-Silyl	- Hexacosanol ($C_{26}H_{54}O$)	382	2.50
	- Octacosanol ($C_{28}H_{58}O$)	410	23.30
	- Triacotanol ($C_{30}H_{62}O$)	438	4.09
	- Dotriacotanol ($C_{32}H_{66}O$)	466	1.49

***β*-sitosterol** (LFD-H3); isolated as colorless needles. The ^{1}H-NMR spectrum showed at δ 3.53 for one proton multiplet, the position and multiplicity of which was indicative of H-3 of the steroid skeleton. The typical H-6 of the steroid skeleton was evident as a multiplet at δ 5.34 that integrated for one proton. The spectrum further showed two methyl singlets at δ 0.68 (H-18) and 1.00 (H-19) (each 3H, *s*). Also three methyl doublets were observed at δ 0.80 (J = 6.6 Hz, H-27), 0.83 (J = 6.5 Hz, H-26) and 0.92 (J = 6.5 Hz, H-21). While the triplet of three proton observed at 0.84

(J = 7.2 Hz) could be assigned to the primary methyl group at H-29. The above spectrum features are in close agreement to those observed for β-sitosterol in literature (Muhit, et al., 2010). β-sitosterol is not previously isolated from *L. loudonii.*

Waxes

The results from GC-MS showed that the constituents of LFD-H1, LFD-H2, LFD-H3, LFD-D1 and LFD-D2-Silyl primarily consisted of odd-numbered saturated straight-chain hydrocarbons (C_{27} to C_{31}), together with saturated fatty acid methyl esters, a fatty aldehyde and primary fatty alcohols with even-numbered carbon chains from C_{26} to C_{32}. The most abundant *n*-alkane was C_{31}. However wax esters of LFD-H2 could not be detected by GC-MS because the temperature of column oven had limited of heat to 320°C, but ^{1}H NMR data confirmed molecular structure and IR spectroscopic data confirmed the functional group. LFD-D2 was silylated before analysed by GC-MS because trimethyl silylation was used to increase volatility for GC-MS.

Plant waxes have previously been reported from the leaf buds and leaves of the Brazilian Mart wax palm, *Copernicia cerifera* (called carnauba wax). The average constituent of the highest quality carnauba wax has been reported as consisting of aliphatic esters (40% w/w), diesters of 4-hydroxy cinnamic acid (21 %w/w), esters of ω-hydroxycarboxylic acids (13% w/w) and free alcohols (12% w/w) (EFSA, 2012). Athukorala, et al. have reported that the major compounds of flax (*Linum usitatissimum*) straw were fatty acids (36 - 49%), fatty alcohols (20 -26%), aldehydes (10-14%), wax esters (5 - 12%), sterols (7 - 9%) and alkanes (4 - 5%). The wax esters primarily consisted of C_{44}, C_{46} and C_{48}. The alkanes consisted of C_{27}, C_{29} and C_{31} (Athukorala et al., 2009). JECFA have reported that plant waxes obtained from stalks of the candelilla plant, *Euphorbia cerifera* and *E. antisyphilitica* (called candelilla wax) consisted of saturated straight-chain hydrocarbons (C_{29} to C_{33}, odd-numbered), together with esters of acids and alcohols with even-numbered carbon chains (C_{28} to C_{34}). The most abundant *n*-alkane, C_{31}, comprises more than 80% of total *n*-alkanes (JECFA, 2005). Waxes are used in cosmetics, lubricants and many other applications (Christie, 2012).

Data analysis of LFD-E1 and LFD-M1

LFD-E1; white amorphous solid; m.p. = 303.0 - 306.0°C (lit. De Silva et al., 1979, 306 - 308°C); $[\alpha]^{28}_{D}$ -0.18 (MeOH, c = 3.46 × 10^{-3} M); IR (ν_{max} KBr): cm^{-1} 3434.5, 2942.6, 2873.9, 1694.1, 1650.0, 1463.0, 1386.6, 1027.2; UV (MeOH, c = 8.5 × 10^{-4} M): λ_{max} = 203 nm (log ε 3.00); ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm): table 4; ESIQ-TOF: *m/z* 455.3537 $[M-H]^-$ for $C_{30}H_{47}O_3$ (Cal. 455.3516); *m/z* = 479.3491 $[M+Na]^+$ for $C_{30}H_{48}O_3Na$ (Cal. 479.3496), corresponding to the structure of 3β-hydroxy-olea-12-en-28-oic acid (oleanolic acid): as shown in figure 5a. The HMBC correlations and full assignments of oleanolic acid were shown in figure 6a.

LFD-M1; pale orange solid; m.p. = 261.0 - 264.0°C (lit. Takagi, et al., 1979, 266 - 267°C); $[\alpha]^{28}_{D}$ -0.35 (MeOH, c = 3.75 × 10^{-3} M); IR (ν_{max} KBr): cm^{-1} 3435.9, 2919.1, 2849.5, 1689.9, 1611.6, 1463.0, 1376.5, 1041.5; UV (MeOH, c = 7.46 × 10^{-4} M): λ_{max} = 203 nm (log ε 2.91); ^{1}H NMR (400 MHz, DMSO-d_6) and ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm): table 5; ESIQ-TOF: *m/z* 455.3475 $[M-H]^-$ for $C_{30}H_{47}O_3$ (Cal. 455.3516); *m/z* = 479.3492 $[M+Na]^+$ for $C_{30}H_{48}O_3Na$ (Cal. 479.3496), corresponding to the structure of 3β-hydroxy-urs-12-en-28-oic acid (ursolic acid): as shown in figure 5b. The HMBC correlations and full assignments of ursolic acid were shown in figure 6b.

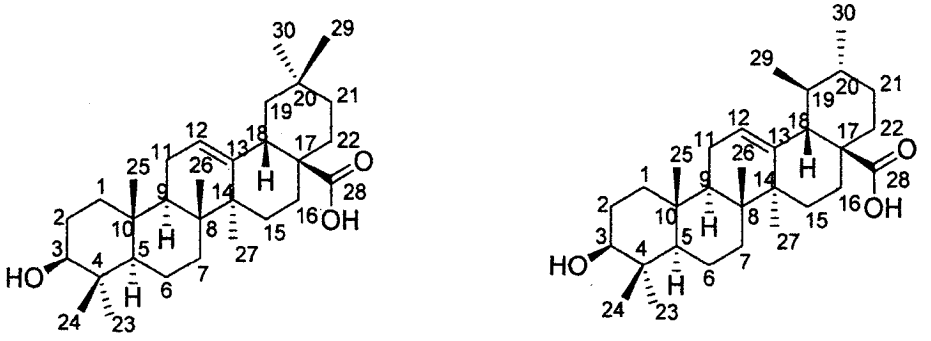

Figure 5 The structures of oleanolic acid (a) and ursolic acid (b)

Table 4 ^{13}C, ^{1}H and HMBC NMR data for LFD-E1 (oleanolic acid) and literature[a]

Atoms	LFD-E1 (oleanolic acid)			Literature OA [a]	
	δ_C	δ_H, multiplicity, *J* in Hz	HMBC	δ_C	δ_H, multiplicity, *J* in Hz
1	38.11	-	-	38.37	-
2	27.33	-	-	27.15	-
3	78.01	2.96, *t*, 7.8	C 1, 2, 23, 24	79.01	3.20, *dd*, 5.0, 11.0
4	38.39	-	-	38.74	-
5	54.88	-	-	55.18	0.75, *t*
6	17.98	-	-	18.27	-
7	32.36	-	-	32.59	-
8	38.95	-	-	39.23	-
9	47.23	-	-	47.6	1.54
10	36.65	-	-	37.05	-
11	22.98	-	-	23.37	-
12	121.70	5.06, *t*, 3.4	C 9, 11, 13, 14, 18	122.61	5.28, *t*, 6.3
13	143.72	-	-	143.26	-
14	41.34	-	-	41.59	-
15	27.66	-	-	27.66	-
16	22.66	-	-	22.91	-
17	45.77	-	-	46.47	-
18	40.76	2.62, *dd*, 4.1, 13.8	C 12, 13, 14, 16, 17, 19, 28	40.98	2.81, *dd*, 3.6, 13.6
19	45.70	-	-	45.84	-
20	30.33	-	-	30.66	-
21	33.55	-	-	33.77	-
22	32.11	-	-	32.40	-
23	27.86	0.77, *s*	C 3, 4, 5, 24	28.08	0.98, *s*
24	15.44	0.56, *s*	C 3, 4, 5, 23	15.52	0.75, *s*
25	14.97	0.69, *s*	C 1, 5, 9,10	15.30	0.91, *s*
26	16.71	0.58, *s*	C 7, 8, 9, 14	17.08	0.77, *s*
27	25.54	0.93, *s*	C 8, 13, 14, 15	25.91	1.13, *s*
28	179.96	-	-	182.66	-

Table 4 (cont.)

Atoms	LFD-E1 (oleanolic acid)			Literature OA [a]	
	δ_C	δ_H, multiplicity, *J* in Hz	HMBC	δ_C	δ_H, multiplicity, *J* in Hz
29	32.80	0.71, *s*	C 19, 20, 21, 30	33.05	0.92, *s*
30	23.27	0.69, *s*	C 19, 20, 21, 29	23.66	0.90, *s*

Table 5 ^{13}C, ^{1}H and HMBC NMR data for LFD-M1 (ursolic acid) and literature[b]

Atoms	LFD-M1 (ursolic acid)			Literature UA [b]	
	δ_C	δ_H, multiplicity, *J* in Hz	HMBC	δ_C	δ_H, multiplicity, *J* in Hz
1	36.65	-	-	39.4	-
2	27.07	-	-	28.1	-
3	77.04	2.99, *dd*, 10.2, 5.7	-	78.1	3.45, *dd*, 10.1, 5.8
4	38.51	-	-	39.1	-
5	54.92	-	-	55.8	-
6	18.12	-	-	18.8	-
7	32.84	-	-	33.6	-
8	40.15	-	-	40.0	-
9	47.15	-	-	48.0	-
10	36.46	-	-	37.3	-
11	22.98	-	-	23.6	
12	124.73	5.12, *br s*	C 9, 14, 18	125.7	5.48, *br s*
13	138.32	-	-	139.3	-
14	41.78	-	-	42.5	-
15	27.67	-	-	28.7	-

Table 5 (cont.)

Atoms	LFD-M1 (ursolic acid)			Literature UA [(b)]	
	δ_C	δ_H, multiplicity, J in Hz	HMBC	δ_C	δ_H, multiplicity, J in Hz
16	23.93	-	-	24.9	(16a) 2.12, *dt*, 12.3, 4.2; (16b) 2.33, *dt*, 12.2, 4.2
17	46.98	-	-	48.0	-
18	52.53	2.09, *d*, 11.7	C 12, 13, 14, 16, 17, 19, 20, 28, 29	53.6	2.63, *d*, 11.3
19	38.64	-	-	39.5	-
20	38.58	-	-	39.4	-
21	30.31	-	-	31.1	-
22	36.46	-	-	37.5	-
23	28.38	0.88, *s*	C 3, 4, 5, 24	28.8	1.24, *s*
24	16.20	0.66, *s*	C 4, 5, 23	16.6	1.02, *s*
25	15.35	0.85, *s*	C 1, 5, 9, 10	15.7	0.88, *s*
26	17.06	0.74, *s*	C 7, 8, 9, 14	17.5	1.05, *s*
27	23.39	1.03, *s*	C 8, 13, 14, 15	23.9	1.22, *s*
28	178.48	-	-	179.9	-
29	17.14	0.80, *d*, 6.4	C 18, 19, 20	17.5	1.00, *d*, 6.4
30	21.21	0.89, *d*, 8.1	C 19, 20, 21	21.4	0.95, *d*, 6.4

Literature in table 4: [(a)] Ragasa and Lim, 2005; 1H NMR (400 MHz, $CDCl_3$), ^{13}C NMR (100 MHz, $CDCl_3$)

Literature in table 5: [(b)] Gohari et al., 2009; ^1H NMR (500 MHz, pyridine-d_5), ^{13}C NMR (125 MHz, pyridine-d_5)

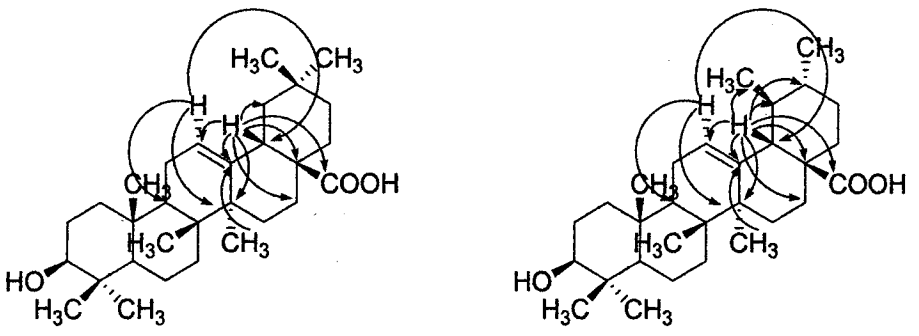

Figure 6 The HMBC correlations and full assignments of oleanolic acid (a) and ursolic acid (b)

Triterpene acids

Oleanolic acid (OA) was isolated as white amorphous solid. ^{1}H-NMR spectrum showed an olefinic proton (H-12) at δ 5.06 as triplet signal with J = 3.4 Hz. The proton geminal to the hydroxyl group (H-3) was evident as triplet at δ 2.96 (J = 7.8 Hz). The methine proton (H-18) appeared as a doublet of doublet at δ 2.62 (J = 13.8, 4.1 Hz). Methyl functionalities attached to saturated carbons appeared as singlets at δ 0.56 - 0.93. The ESIQ-TOF showed $[M-H]^-$ peak at *m/z* 455.3537, corresponding to the molecular formula ($C_{30}H_{48}O_3$) for OA. The spectroscopic data of this compound were identical to the data of Ragasa and Lim in 2005 (Ragasa and Lim, 2005).

Ursolic acid (UA) was isolated as a pale orange solid. The ^{1}H-NMR spectrum showed an olefinic proton (H-12) at δ 5.12 as broad singlet signal. The proton geminal to the hydroxyl group (H-3) was evident as doublet of doublet at δ 2.99 (J = 10.2, 5.7 Hz). The methine proton (H-18) appeared as a doublet at δ 2.09 (J = 11.7 Hz). Five tertiary methyl functionalities appeared as singlets at δ 0.66, 0.74, 0.85, 0.88 and 1.03, while the signals at δ 0.80 (3H, *d*, J = 6.4 Hz) and 0.89 (3H, *d*, J = 8.1 Hz) were indicative of an ursane skeleton. The ESIQ-TOF showed $[M-H]^-$ peak at *m/z* 455.3475, corresponding to the molecular formula ($C_{30}H_{48}O_3$) for UA. It was the major constituent of this extract.

OA and UA are derivatives of pentacyclic triterpene acids. Both molecular structures are similar, differing only at the sites of the methyl group (methyl group at C-19 is UA, at C-20 is OA) on the E ring.

OA and UA have not previously been reported as a constituent of *L. loudonii*. OA has been isolated from other species in the Lythraceae family, including from leaves of *L. speciosa* (Hou, et al., 2009).

Screening bioactivity of OA and UA

OA and UA have anti-malarial (IC_{50} 8.8 μg/mL and 1.0 μg/mL; Sairafianpour, et al., 2003, Filho, et al., 2009 respectively), anti-HIV (IC_{50} 47.8 μg/mL and 14.3 μg/mL; Kashiwada, et al., 2000) and anti-TB (MIC 50 μg/mL and 12.5 μg/mL; Tanachatchairatana, et al., 2008) activities. The DCM extract from *L. loudonii* fruit has the anti-TB activity (MIC 100 μg/mL; Boonphong, et al., 2003) that is less effective than OA and UA.

Antitumor activity of UA has been reported for several human cancer cell lines (liver, prostate, breast, skin, melanoma, brain, thyroid, gastric, lung, ovarian and colon) (Khoo, 2011). The report of the antitumor activity of OA showed the moderate cytotoxic activity against the four cancer cell lines (liver, cervix, breast and colon) (Hasshem, et al., 2012). Moreover OA exhibits antiviral, anti-inflammatory, hepatoprotective and anti-hyperlipidemic effects. Chinese has used OA as a medicine to treat liver disorders for over 20 years (Wang, et al., 2010). In addition Xi et al. reported the conventional formulations of OA tablets and capsules (Xi, et al., 2009).

OA and UA have been reported to possess diverse biological and pharmaceutical activities, such as anti-inflammatory and hepatoprotective effects (Liu, 1995) including antioxidant activity (Wang, et al., 2010).