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Waxes and Triterpene acids from *Lagerstroemia loudonii* fruit

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ABSTRACT

Dichloromethane crude extracts from the *Lagerstroemia loudonii* fruit were purified by column chromatography and the isolated compounds were identified by spectroscopic methods, particularly by extensive Nuclear Magnetic Resonance (NMR) techniques. The isolated compounds were waxes, oleanane-type triterpene acid; oleanolic acid (OA) and ursan-type triterpene acid; ursolic acid (UA). This is the first report on the isolation of waxes and isomeric triterpene acids as OA and UA from this plant.

Key words: Lagerstroemia loudonii, oleanolic acid, ursolic acid, waxes

INTRODUCTION

Lagerstroemia loudonii (Lythraceae) is a tropical plant mainly distributed in the northern part of Thailand. This genus has 18 species found in mixed forest (Smitinand, 2001). Some species are used for a local Thai herbal medicine such as *L. floribunda* and *L. loudonii* barks are used for treating diarrhea, while *L. speciosa* leaf is used for diuretic and diabetes. The wide range of pharmaceutical activity of *L. speciosa* has been studied, for example, control level in total hepatic lipid contents in genetically diabetic mice (type II, KK-A^y) (Suzuki *et al*., 1999), reduce blood pressure, lower blood glucose levels, antivirus, antifungal, antibacterial, anticancer (Punyaprabhasara, 2000) and antidiabetic activity in humans (Judy *et al*., 2003). Boonphong *et al*. reported that dichloromethane and methanol extracts from *L. loudonii* have antioxidant, anti-TB and anti-malarial activities. Especially the dichloromethane extract from *L. loudonii* fruit has the anti-TB (MIC 100 μg/mL) and the anti-malarial activity (EC_{50} 2.20 μg/mL) which are more effective than the methanol extract from the same part of plant (Boonphong, *et al*., 2003). The isolated compounds of the methanol and dichloromethane extracts from flower of *L. loudonii*

are ellagitannins, which has high antioxidant activities (DPPH free radical scavenging assay) (Malaisree *et al*., 2006). The preliminary tests of four species of the genus *Lagerstroemia* by Prasitpan *et al*. showed that there were only trace amount of alkaloids in the leaf of *L. speciosa, L. macrocarpa, L. loudonii, L. duperreana* and in the bark of *L. duperreana*. Flavonoids had not been found in the leaf and the bark of *L. duperreana*. Trace amounts of flavonoids were found in the leaf of *L. speciosa* but large amounts were discovered in the leaves of *L. macrocarpa* and *L. loudonii*. In addition steroids were found in all leaf and bark samples. (Prasitpan *et al*., 1988)

However, the study on the chemical constituents of *L. loudonii* fruit extracts has never been reported yet. In this work, the compounds from *L. loudonii* fruit were extracted and isolated by column chromatography. The identification of the chemical structure of isolated compounds were done by spectroscopic techniques.

MATERIALS AND METHODS

General Experimental Procedures

UV spectra were recorded on a Jasco V-650 spectrophotometer. Melting points were measured on a Stuart Scientific UK melting point apparatus. Optical rotations were measured on a ADP200 polarimeter. The FT-IR spectra were measured on a Perkin-Elmer Frontier spectrophotometer. ^{1}H and ^{13}C, DEPT (135), ^{1}H-^{1}H COSY, HMQC and HMBC experiments were carried out on a Bruker AV400 NMR spectrometer, operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. ESIQ-TOF mass spectra were obtained from a 6540 UHD Accurate-Mass Q-TOF LC/MS Agilent Technologies USA. Column chromatography (CC) was performed using Merck silica gel 60H for TLC (< 55μm; Darmstadt, Germany; 1.07736.1000) (CC equip with low pressure air pump), Sephadex LH-20 (Amersham Pharmacia Biotech AB, Uppsala, Sweden), Thin-layer chromatography (TLC) was run using Merck silica gel F-254 (1.05554.0001).

The analyses were performed on a 5973 Network MSD Agilent, a 6890N Network GC system Agilent and Agilent 19091S-433 HP-5MS capillary column (30 m × 0.25 mm, a film thickness of 0.25 μm). The GC was operated in the constant flow mode of 1.5 mL/min with helium as a carrier gas. The column oven was temperature programmed: 70°C (1 min), then 50°C/min to 240°C, 1°C/min to 320°C and held at 320°C for 10 min. The temperatures of ion source and quadrupole were 230°C and 150°C, respectively. The quadrupole was scanned in the 25-675 *m/z* range (Urbanova *et al*., 2012).

Solvent

Hexane, dichloromethane, chloroform, ethyl acetate, acetone and methanol were obtained from Mallinckrodt Chemicals (USA). Toluene was obtained from RCI Labscan (Thailand). Chloroform-D and acetone-d_6 (99.9%D) were obtained from Wilmad LabGlass (USA). Methyl sulfoxide-d_6 (99.9%D) was obtained from ALDRICH (USA). Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylsilyl chloride (BSTFA/TMSCl) was obtained from Acros Organics (USA).

Plant material

Lagerstroemia loudonii fresh fruits were collected in Naresuan University, Phitsanulok province, Thailand, during March to April 2013. Voucher specimens (003507) were deposited at Department of Biology, Faculty of Science, Naresuan University.

Extraction

The fresh fruits of *L.loudinii* (4 kg) were cracked and macerated in methanol (5.0 L × 2, each) at room temperature for one week. Then the solution was percolated, and the combined filtrates were evaporated under vacuum to produce a brown-gummy methanol extract. The remaining fruit was macerated in dichloromethane (DCM, 5.0 L × 2, each) at room temperature for another one week. Then the solution was percolated. After that the combined filtrates were evaporated under vacuum to produce 14.37 g (0.36%) of a green-powder DCM extract.

The DCM extract (LFD, 10.0 g) was suspended in non polar solvent to more polar solvent that is hexane (500 mL), DCM (250 mL), EtOAc (250 mL) and acetone (250 mL). Each the soluble part was evaporated and labeled as fraction LFD-H (4.10 g), LFD-D (1.30 g), LFD-E (0.75 g) and LFD-A (0.61 g), respectively. The precipitate was suspended in MeOH (250 mL). The soluble part was evaporated but no residue was found. Therefore, the precipitate was labeled as fraction LFD-M (2.93 g)

Isolation by column chromatography

Fraction LFD-H (4.10 g) was separated by silica gel CC (3 cm × 10 cm) with a solvent gradient of 100% hexane to 15% acetone-DCM to afford twenty-six subfractions (h1-h26). **LFD-H1** (0.72 g, 17.6%) was obtained from subfraction h3. **LFD-H2** (0.88 g, 21.5%) was obtained from subfraction h8. Mix of subfraction h11 to h18 (0.38 g) was fractionated by CC on silica gel (2 cm × 10 cm) eluted with 100% hexane to 50% DCM-hexane to afford sixty-one subfractions (hm1-hm61). **LFD-H3** (7.3 mg, 0.18%) were obtained from hm38.

Fraction LFD-D (0.89 g) was separated by silica CC (3 cm × 10 cm) with a solvent gradient of 20% DCM-hexane to 100% DCM, 1% EtOAc-DCM to 10% EtOAc-DCM to afford twenty-five subfractions (d1-d25). **LFD-D1** was obtained from subfraction d3 (8.80 mg, 0.10%). **LFD-D2** was obtained from subfraction d17 (4.20 mg, 0.05%).

Fraction LFD-E (0.75 g) and LFD-A (0.61 g) were combined and separated by silica CC (3 cm × 10 cm) with a solvent gradient of 1% Acetone-$CHCl_3$ to 100% acetone to afford twenty-five subfractions (e1-e25). **LFD-E1** was obtained from subfraction e20 (0.14 g, 10.3%)

Fraction LFD-M was washed with hexane several times. **LFD-M1** (2.92 g, 99.6%) was obtained from the precipitate.

Analytical procedure

The structures of LFD-H1, LFD-H2, LFD-H3, LFD-D1, LFD-D2, LFD-E1 and LFD-M1 were determined by 1D nuclear magnetic resonance (NMR) spectroscopy. Structures of LFD-E1 and LFD-M1 were confirmed by 2D NMR spectroscopy, melting point, optical rotations, IR spectroscopy, UV-Vis spectrophotometry and ESIQ-TOF mass spectrometry.

Silylation

The silylation of LFD-D2 was carried out based on Inarkar and Lele report (Inarkar and Lele, 2012). LFD-D2 was trimethylsilylated using 200 μL of BSTFA/ TMSCl and 100 μL of toluene. The mixture was stirred at 75°C for 30 min. The reaction mixture was cooled and concentrated under nitrogen flush. The residue was redissolved in hexane, and the mixture was labeled as **LFD-D2-Silyl**.

Gas Chromatography-Mass Spectrometry Analysis

LFD-H1, LFD-H2, LFD-H3, LFD-D1 and LFD-D2-Silyl were analyzed and identified using GC-MS. The compounds were identified by comparison of fragmentation patterns in mass spectra with those of Wiley7n.1 library.

RESULT AND DISCUSSION

Data analysis of 5 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; ^{1}H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^{1}H 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, 1175.2; ^{1}H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^{1}H NMR profile of wax ester reported in the reference. (Pouchert *et al*., 1993)

LFD-H3; white amorphous solid; IR (ν_{max} neat): cm^{-1} 2917.3, 2849.6, 1743.4, 1170.8; ^{1}H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^{1}H 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, 1735.3; ^{1}H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^{1}H 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, 1062.3; ^{1}H NMR (400 MHz, $CDCl_3$) data of this fraction were in good agreement with ^{1}H NMR profile of fatty alcohol reported in the reference. (Pouchert *et al*., 1993)

The chemical composition analyses of 5 fractions were further investigated by GC-MS and the results were showed in table 1.

Table 1 Chemical composition analysis of LFD-H1, LFD-H2, LFD-H3, 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-H3	-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

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, 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 2; 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 1.

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 2; 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 1.

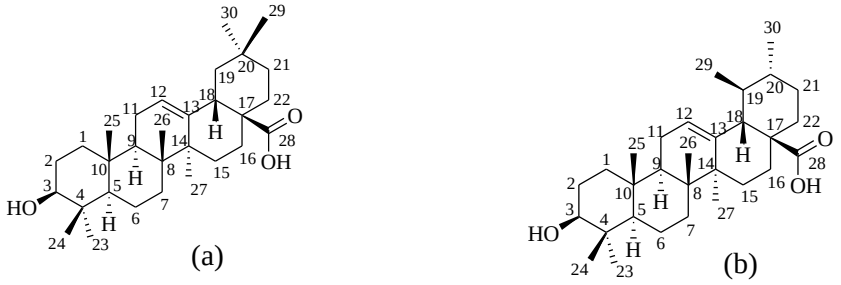

Figure 1 The structures of oleanolic acid (a) and ursolic acid (b)

Table 2 ^{13}C and ^{1}H NMR spectral data for LFD-E1 (oleanolic acid) and LFD-M1 (ursolic acid)

Atoms	LFD-E1(oleanolic acid)		LFD-M1(ursolic acid)	
	δ_C	δ_H, multiplicity, *J* in Hz	δ_C	δ_H, multiplicity, *J* in Hz
1	38.1	-	36.7	-
2	27.3	-	27.1	-
3	78.0	2.96, *t*, 7.8	77.0	2.99, *dd*, 10.2, 5.7
4	38.4	-	38.5	-
5	54.9	-	54.9	-
6	18.0	-	18.1	-
7	32.4	-	32.8	-
8	39.0	-	40.2	-
9	47.2	-	47.2	-
10	36.7	-	36.5	-
11	23.0	-	23.0	-
12	121.7	5.06, *t*, 3.4	124.7	5.12, *br s*
13	143.7	-	138.3	-
14	41.3	-	41.8	-
15	27.7	-	27.7	-
16	22.7	-	23.9	-
17	45.8	-	47.0	-
18	40.8	2.62, *dd*, 13.8, 4.1	52.5	2.09, *d*, 11.7
19	45.7	-	38.6	-
20	30.4	-	38.6	-
21	33.6	-	30.3	-
22	32.1	-	36.5	-
23	27.9	0.77, *s*	28.4	0.88, *s*
24	15.4	0.56, *s*	16.2	0.66, *s*
25	15.0	0.69, *s*	15.4	0.85, *s*
26	16.7	0.58, *s*	17.1	0.74, *s*
27	25.5	0.93, *s*	23.4	1.03, *s*
28	180.0	-	178.5	-
29	32.8	0.71, *s*	17.1	0.89, *d*, 8.1
30	23.3	0.69, *s*	21.2	0.80, *d*, 6.4

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).

CONCLUSIONS

The chemical constituents of *L. loudonii* were successfully isolated and studied for the biological activities. Triterpene acids exhibited anti-TB, anti-malarial, anti-HIV and antitumor activities. This is the first report on the isolation of waxes, OA and UA from this plant. Moreover, *L. loudonii* fruits are new source to produce waxes and UA which has the anti-TB and anti-malarial activities.

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