ORIGINAL ARTICLES

Bioactive Constituents from the Twigs of *Sonneratia alba*

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ABSTRACT

Three pentacyclic triterpenoids: lupeol **[1],** oleanolic acid **[2],** and betulinic acid **[3]** were isolated from the twigs of *Sonneratia alba* together with 2,6-dimethoxy-*p*-benzoquinone **[4],** a mixture of stigmasterol **[5]** and β-sitosterol **[6].** Their structures were elucidated by analyses of their 1D and 2D NMR, and their physical and spectral data were compared with those reported in the literature. Amongst these isolates, compounds **[2,3]** exhibit antimycobacterial activity with MIC values of 25 and 50 μg/ml respectively. In addition, compound **[4]** exhibited antimalarial activity against *P. falciparum* with an IC_{50} value of 3.08 μg/ml.

Key words: *Sonneratia alba* – Sonneraceae – Triterpenoids – Quinone – Antimalarial - Antimycobacterial

INTRODUCTION

Sonneratia alba

Sonneratia alba is a mangrove plant belonging to Family Sonneraceae (locally called "Lampoo Talay") and is distributed widely in the coastal areas of South East Asia and the Indian Ocean. *S. alba* is used in traditional Thai medicine for the treatment of diarrhea, healing of wounds and fever (1). This plant has not previously been investigated chemically. As part of our continuing chemical studies of Thai medicinal plants (2-4), a preliminary investigation showed that the dichloromethane extract of the twigs of *S. alba* inhibited *Plasmodium falciparum* with the IC_{50} value of 6.70 μg/ml. In this paper, we report the chromatographic separation of compounds **[1-6]** from the dichloromethane extract of the twigs of *S. alba* and the biological evaluations of these isolates. The structures of compounds **[1-6]** were determined by spectroscopic methods, and their spectral data were compared with those reported in the literature. Compounds **[1-6]** were evaluated for their antimycobacterial activity against *Mycobacterium tuberculosis* H37Ra and antiplasmodial activity against *Plasmodium falciparum*.

RESULTS AND DISCUSSION

The dichloromethane extract of the twigs of *S. alba* was separated by successive column and thin layer chromatography with various eluting systems to give three known pentacyclic triterpenoids **[1-3]**, a quinone **[4]** and two steroids **[5,6]**.

Lupeol [1] was isolated as colourless crystals. The IR spectrum exhibited an absorption band at 3436 cm^{-1} indicating the presence of hydroxyl group. The mass spectrum displayed a $[M]^+$ ion at *m/z* 426, analysing for $C_{30}H_{50}0$. The ^1H NMR spectrum **(Table 1)** of **[1]** showed signals for six tertiary methyl groups at *d* 0.76, 0.79, 0.83, 0.94, 0.97 and 1.03, and further displayed an isopropenyl side chain by signals at *d* 1.68 (3H, s, H-30), 4.57 and 4.69 (each 1H, d, H-29α and H-29*b*) manifesting its relation with lupane-type of triterpenoids. Its physical and spectroscopic (IR, ^1H and ^{13}C NMR) data were the same as those reported for lupeol in the literature (5-7). Its identity was also confirmed by comparison (co-TLC and superimposable IR) with an authentic sample. Compound **[1]** was therefore identified as lupeol.

Oleanolic acid [2] was isolated as a white solid with a molecular formula of $C_{30}H_{46}O_3$. The IR spectrum showed the presence of a hydroxyl group at 3420 cm^{-1}. The ^1H NMR spectrum **(Table 1)** revealed the presence of seven tertiary methyl groups [*d* 0.75, 0.78, 0.90, 0.91, 0.93, 0.99 and 1.14 (each 3H, s)]. The ^{13}C NMR spectrum revealed 30 carbon signals which were shown by a DEPT experiment to be seven methyls, nine methylenes, five methines, five quaternary carbons, one carboxylic acid, and two olefinic carbons revealing that **[2]** is a triterpenic acid having five rings. The singlet signals for seven methyls in the ^1H NMR spectrum and olefinic signals at 122.64 and 143.55 corresponding respectively to C-12 and C-13 in the ^{13}C NMR spectrum indicated the presence of Δ^{12}-oleanane skeleton in compound **[2].** This spectral feature indicated that **[2]** belongs to an oleanane-type triterpene having a carboxylic function. The compound **[2]** was identified unambiguously as oleanolic acid by comparison with an authentic sample and the reported oleanolic acid (8).

Betulinic acid [3] was isolated as colourless crystals, whose molecular formula $C_{30}H_{48}O_3$ was established by its EIMS mass spectrum. Its IR spectrum indicated the absorption for hydroxyl (3443 cm^{-1}) and carboxyl groups (1688 cm^{-1}). The ^{13}C NMR spectrum **(Table 1)** revealed 30 carbon signals which were shown by a DEPT experiment to be five methyls, five quaternary carbons, one carboxylic acid, and two olefinic carbons revealing that **[3]** is a triterpenic acid having five rings. The ^1H NMR spectrum of **[3]** showed the signals for five tertiary methyl groups at *d* 0.75, 0.82, 0.93, 0.97, 0.98, one isopropenyl moiety at *d* 1.69, 4.62 and 4.75 indicating a lupane-type skeleton. Compound **[3]** was identified as betulinic acid by direct comparison of its physical and spectral data with the reported values (9).

2,6-Dimethoxy-*p*-benzoquinone [4] was isolated as yellow needles. The UV spectrum showed an absorption band at 288 nm indicating the presence of an ∝, β-unsaturated keto function. The IR spectrum showed an absorption band at 1696 cm^{-1} indicating the unsaturated carbonyl group. The ^1H NMR spectrum of this compound showed a singlet signal of methoxy protons at *d* 3.81 together with a singlet signal of olefinic protons at *d* 5.82. The ^{13}C NMR spectrum revealed four signals for eight carbon atoms at *d* 187.50 (C=O), 172.00 (C=O), 157.33 (2xC), 107.42 (2xC), 56.52 (2xC). Compound **[4]** was identified as 2,6-dimethoxy-*p*-benzoquinone by comparisons of its melting point and spectroscopic (IR, ^1H and ^{13}C NMR spectra) data with authentic samples.

Stigmasterol [5] and *b*-Sitosterol [6] were isolated as colourless crystals with a melting point at 135-136°C. The IR spectrum showed absorption bands at 3260 and 1664 cm^{-1} indicating the presence of hydroxyl and olefinic groups in the molecule. The ^{1}H and ^{13}C NMR spectra were consistent with those reported for stigmasterol and β-sitosterol (10). Compounds **[5,6]** were therefore identified as a mixture of stigmasterol and β-sitosterol in a ratio of 1:3.

Compounds [1-3] showed no activity against *P. falciparum* while compounds **[2,3]** exhibited antimycobacterial activity with MIC values of 25 and 50 *m*g/ml respectively **(Table 2)**. It should be noted that the quinone [4] was the only active (IC_{50} 3.08 *m*g/ml) compound against *P. falciparum*.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on an electrothermal melting point apparatus and were uncorrected. UV spectra were measured with an UV SPECCORD S100 spectrophotometer, and IR spectra were recorded on a Perkin-Elmer 1750 FTIR spectrophotometer. The ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance DPX-300 MHz and 500 MHz Varian Unity INOVA spectrometers with TMS as internal standard. Chemical shifts were recorded in part per million (*d*) in $CDCl_3$. Quick column chromatography and column chromatography were carried out on a silica gel 60 F_{254} (Merck) and silica gel 100 respectively. Silica gel 60 F_{254} precoated aluminum plates (0.2 mm, Merck) were used for TLC analysis; detection was performed by spraying with 5% H_2SO_4 in ethanol and 1% vanillin in ethanol followed by heating at 100 -110°C for 5 min.

Plant Material. The stems of *S. alba* were collected at the Mangrove Research Station in Nakhon Si Thammarat Province, Thailand. A voucher specimen has been deposited at Walailak University.

Extraction and Isolation. Air-dried and powdered stems of *S. alba* (3.0 kg) were extracted with dichloromethane at room temperature. The dichloromethane extract was dried under reduced pressure to give the crude extract (9.20 g). The crude extract was then subjected to column chromatography on silica gel and eluted initially with hexane enriched with dichloromethane, ethyl acetate, followed by increasing amounts of methanol in ethyl acetate and finally with methanol. Each fraction was monitored by TLC; fractions that appeared similar on TLC were combined to yield 21 fractions, F1-F21. Fraction F6 (1.021 g) was rechromatographed on a silica gel flash column chromatography followed by preparative TLC with 10% hexane in dichloromethane to give **[1]** (0.096 g). Fraction F14 (0.943 g) was further purified by flash column chromatography to give **[4]** (0.008 g) and a mixture of **[5,6]** (0.082 g). Fraction F15 (2.256 g) was similarly purified by flash column chromatography to afford **[2]** (0.216 g) and **[3]** (0.084 g).

Lupeol [1]. Colourless crystals, mp. 200-202°C. IR (KBr) ν_{max} 3436, 2945, 2872, 1265 and 740 cm^{-1}. EIMS, *m/z* 426 $[M]^+$ (calcd. for $C_{30}H_{50}O$, 426.3859). ^{1}H and ^{13}C NMR data, see **Table 1.**

Oleanolic acid [2]. White solid, mp. 205-208°C. IR (KBr) ν_{max} 3420, 2927, 2867, 1093, 1492, 1049 and 824 cm^{-1}. EIMS, *m/z* 454 $[M]^+$ (calcd. for $C_{30}H_{46}O_3$, 454.3447). ^{1}H and ^{13}C NMR data, see **Table 1**.

Betulinic acid [3]. Colourless crystals, mp. 254-256°C. IR (KBr) ν_{max} 3443, 2944, 2871, 1688, 1452 and 883 cm^{-1}. EIMS *m/z* 456 $[M]^+$(calcd. for $C_{30}H_{48}O_3$, 456.3603). ^1H and ^{13}C NMR data, see **Table 1**.

Table 1. ^1H and ^{13}C NMR spectral data for compounds **[1-3]**

Position	1		2		3	
	d_C	d_H	d_C	d_H	d_C	d_H
1	38.84		38.38		38.87	
2	27.39		23.38		27.39	
3	78.99	3.20 (dd, *J*=4.5, 11.0 Hz)	79.01	3.22 (dd, *J*=4.5, 11.5 Hz)	79.02	3.20 (dd, *J*=5.0, 11.5 Hz)
4	37.14		38.74		38.40	
5	55.26	0.69 (m)	55.18		55.32	0.67 (m)
6	18.29	1.50 (1H, m) 1.40 (1H, m)	12.28		18.28	
7	34.24		32.40		34.30	1.42 (m)
8	40.80		39.24		42.43	
9	50.40		47.60		50.49	1.27 (m)
10	42.80*		37.05		37.20	
11	20.90		22.94		20.84	
12	25.10		122.64	5.29 (t, *J*=3.5 Hz)	30.55	
13	38.01		143.55		38.69	2.19 (dt, *J*=3.0, 12.5 Hz)
14	42.98*		41.60		40.68	
15	27.42		27.66		29.70	
16	35.56		25.90		25.48	
17	38.67		46.47		56.34	
18	48.27		41.02	2.82 (dd, *J*=4.5, 14.0 Hz)	49.25	1.62 (t, *J*=11.5 Hz)
19	47.97	2.38 (dt, *J*=5.5, 11.0 Hz)	45.85		46.90	3.01 (dt, *J*=4.5, 10.5 Hz)
20	150.98		30.66		150.42	
21	29.81	1.92 (1H, m), 1.26 (1H, m)	33.77		20.84	1.38 (m)
22	39.98		32.60		32.16	2.25 (td, *J*=3.0, 13.0 Hz)
23	27.97	0.97 (s)	28.08	0.99 (s)	15.35	0.75 (s)
24	15.36	0.76 (s)	15.53	0.78 (s)	27.99	0.97 (s)
25	15.95	0.83 (s)	15.31	0.91 (s)	16.13	0.82 (s)
26	16.10	1.03 (s)	17.07	0.75 (s)	16.03	0.93 (s)
27	14.53	0.94 (s)	27.16	1.14 (s)	14.69	0.98 (s)
28	17.98	0.79 (s)	182.21	-	180.02	-
29	209.31	4.69 (d, *J*=2.5 Hz), 4.57 (s)	33.05	0.90 (s)	109.71	4.75 (d, *J*=2.0 Hz), 4.62 (s)
30	19.29	1.68 (s)	23.05	0.93 (s)	19.14	1.69 (s)

* Assignment with the same superscripts may be interchanged

2,6-Dimethoxy-*p*-benzoquinone [4]. Yellow crystals, mp. 240°C. IR (KBr) ν_{max} 1696, 1594 cm^{-1}. EIMS *m/z* 169.0202 [M+H]; (calcd. for $C_8H_9O_4$, 169.0498). ^1H NMR *d* 5.86 (s, 2H), 3.83 (s, 6H) and ^{13}C NMR *d* 186.86, 176.72, 157.34, 107.54 and 56.52.

Stigmasterol and *b*-Sitosterol [5,6]. Colourless crystals, mp. 135-136°C. IR (KBr) ν_{max} 3435, 2973, 2937, 2842 and 1637cm^{-1}. ^1H NMR *d* 5.40-5.39 (m), 5.19 (dd, *J*=15.0, 8.5 Hz), 5.06 (dd, *J*=15.0, 8.5 Hz), 3.59-3.53 (m), 2.33 (ddd, *J*=13.0, 5.0, 2.0 Hz), 2.30-2.24 (m), 2.28 (qd, *J*=11.5, 2.5 Hz), 2.30-2.24 (m), 2.08-1.98 (m), 1.90-1.84 (m), 1.76-1.67 (m), 1.65-1.45 (m), 1.34-1.21 (m), 1.06 (d, *J*=7.5 Hz), 1.04 (s), 0.96 (d, *J*=6.5 Hz), 0.89 (d, *J*=4.0 Hz), 0.88 (d, *J*=1.5 Hz), 0.86 (d, *J*=4.0 Hz), 0.85 (d, *J*=1.5 Hz), 0.84 (d, *J*=6.5 Hz), 0.73 (s), 0.71 (s).

Table 2. Biological activities of compounds **[1-6]**

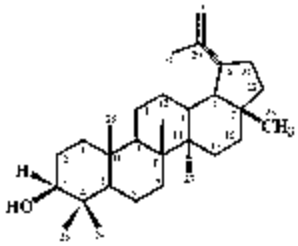
Compounds	Antiplasmodial IC_{50} (*m*g/ml)	Antimycobacterial MIC (*m*g/ml)
1	inactive[a]	inactive[b]
2	inactive[a]	25
3	inactive[a]	50
4	3.08	inactive[b]
5 and **6**	inactive[a]	inactive[b]

[a] *inactive at > 10 mg/ml*
[b] *inactive at > 200 mg/ml*

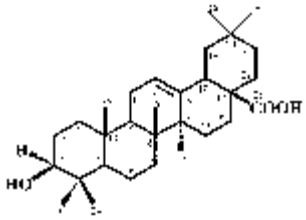
Bioassays. The malarial parasite, *Plasmodium falciparum* (K1, multidrug resistant strain), was cultured according to the method of Trager and Jensen (12). Quantitative assessment of malarial activity *in vitro* was determined by means of the microculture radioisotope technique based upon the method described by Desjardin et al (13). The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the *in vitro* uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 1 *m*g/ml was observed for the standard compound, artemisinin, in this test system. The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA) (14). The standard drugs, isoniazid and kanamycin sulfate, used as reference compounds for the antimycobacterial assay, showed MIC values of 0.05 and 2.5 *m*g/ml respectively in the test systems.

ACKNOWLEDGMENTS

We thank the Biodiversity Research and Training Program (BRT), Taiyo Group of Japan, the Shell Centennial Education Foundation, and Walailak University Fund for financial support. Bioassay Research Facility of BIOTEC is also gratefully acknowledged for bioactivity tests.



Lupeol [1]



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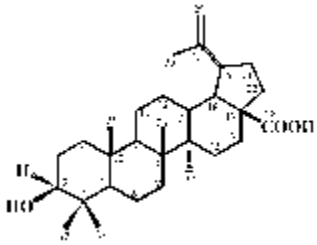

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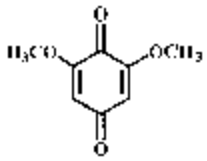
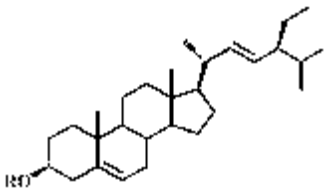
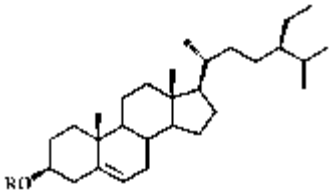


2,6-Dimethoxy -*p*-benzoquinone [4]



Stigmasterol [5]



***b*-Sitosterol [6]**

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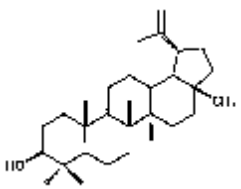
บทคัดย่อ

กาญจุรีย์ ไชยเดช[1] หทัยชนก วงษ์เทพ สุรสีห์ วัฒนวิกย์กิจ และ ก้าน จันทร์พรหมมา[2]

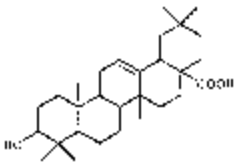
องค์ประกอบทางเคมีและฤทธิ์ทางชีวภาพของสารจากกิ่งลำพูทะเล

ส่วนสกัดหยาบไดคลอโรมีเทนของกิ่งลำพูทะเล นำมาแยกและทำให้บริสุทธิ์ด้วยวิธีทางโครมาโทกราฟี สามารถแยกสารที่ทราบโครงสร้างแล้ว จำนวน 6 สาร ซึ่งเป็นสารประเภท Pentacyclic triterpenoids จำนวน 3 สาร คือ Lupeol **[1]**, Oleanolic acid **[2]** และ Betulinic acid **[3]** สารประเภท Benzoquinone จำนวน 1 สาร คือ 2,6-Dimethoxy-*p*-benzoquinone **[4]** และอีกจำนวน 2 สาร ซึ่งเป็นสารผสมของ Stigmasterol **[5]** และ β-Sitosterol **[6]** โดย **[2]** และ **[3]** มีฤทธิ์ต้านเชื้อวัณโรค (antimycobacterial) ที่ MIC มีค่าเป็น 25 และ 50 *m*g/ml ในขณะที่ **[4]** มีฤทธิ์ต้านเชื้อมาลาเรีย (antimalarial against *P. falciparum*) ที่ IC_{50} มีค่าเป็น 3.08 *m*g/ml

โครงสร้างของสารทุกตัว วิเคราะห์โดยใช้ข้อมูลทางสเปกโทรสโกปี โดยเฉพาะ 1D และ 2D NMR สเปกโทรสโกปี นอกจากนี้ ยังยืนยันโครงสร้างจากข้อมูลที่มีการรายงานแล้ว



Lupeol **[1]**



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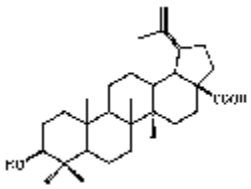

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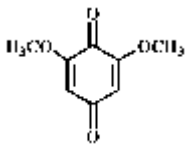
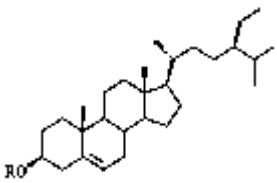
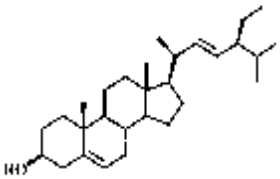


2,6-Dimethoxy-*p*-benzoquinone **[4]**



Stigmasterol **[5]**



b-Sitosterol **[6]**

[1] ศูนย์เครื่องมือวิทยาศาสตร์และเทคโนโลยี สำนักวิชาวิทยาศาสตร์[2] และสำนักวิชาสหเวชศาสตร์และสาธารณสุขศาสตร์[3] มหาวิทยาลัยวลัยลักษณ์ อำเภอท่าศาลา จังหวัดนครศรีธรรมราช 80160