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Tectona grandis Callus Produces Antibacterial Triterpene Acids Not Detected in the Intact Plant

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Abstract – Preliminary antibacterial assay data that the *Tectona grandis* callus extract showed more antibacterial activity against *E. coli* and *B. subtilis* than the leaf extract led the authors to isolate the following antibacterial compounds from the callus. A mixture (**3**) of 2α,3β-dihydroxy-olean-12-en-28-oic acid (**3a**) and 2α,3β-dihydroxy-urs-12-en-28-oic acid (**3b**) exhibited the most potent antibacterial activity against both bacteria. The other 3 compounds, in the decreasing order of the activity, were identified as 2α,3α-dihydroxy-urs-12-en-28-oic acid (**2**), betulinic acid (**1**), and 2α,3α, 23-trihydroxy-urs-12-en-28-oic acid (**4**). The antibacterial compounds (**2, 3a, 3b** and **4**) were not detected or occurring in small quantities in the intact tissue, while they were observed in the callus. Only the less active compound **1** was present more abundantly in intact tissues than the callus.

Keywords – *Tectona grandis* callus, triterpene acids, antibacterial compounds, oleanene, lupene, ursene.

Introduction

Plant cell culture has been considered since 1970s to be a potential technique for producing natural secondary metabolites *in vitro*. The possibility of using plant cell culture for secondary metabolite production has been investigated for over 25 years, with potential advantages of producing metabolites under environmentally controlled conditions, free from diseases and pests. Perhaps, one of the important advantages of using cell culture is a potential for producing useful compounds not occurring in the parent plant (Fowler, 1983; Wakayama *et al.*, 1994). However, in spite of extensive optimization of culture conditions, cultured cells mostly synthesized and accumulated specific secondary metabolites at lower levels than the intact plant. *Tectona grandis* ('teak'), a tropical tree belonging to the family of Verbenaceae, is widely cultivated in many parts of the tropics for the main purpose of supplying good timbers which are remarkably durable due to its significant resistance to termites. The extract of young leaves was reported to be a folk medicine for cholera (Heyne *et al.*, 1987). While many chemical studies have been done on the intact plant of *T. grandis*, little

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information is available on the cultured cells. One of the available literatures concerns production of quinone in stem tissues grown *in vitro* (Dhruva *et al.*, 1972). The literature survey, which indicated that little information was available on the production of secondary metabolites in cultured cell of *T. grandis*, led the author to investigate secondary metabolite production in cultured cell of *T. grandis* with expectation to find novel products not observed in the parent plant. The use of the extract of the *T. grandis* leaves for treatment for cholera might reflect that the leaves contained antimicrobial compounds. Comparison between antimicrobial activities of the solvent extracts of the leaves and callus showed that the callus extract had stronger antibacterial activity than the leaf extract. Among the EtOAc, H_2O, and 1-BuOH fractions obtained from the methanol extract of the callus, the EtOAc fraction exhibited the strongest antibacterial activity. Based on these preliminary data, isolation and characterization of the antibacterial compounds from the callus have been carried out.

Experimental

General – Mps: uncorrected; MS: JEOL SX-102A; IR: as KBr pellets with Nicolet 710 FT-IR; NMR: Varian VXR-500 at 500 MHz (^{1}H) and 125 MHz (^{13}C); HPLC: Waters LC Module I equipped with U6K injector; Specific rotation: at ambient temperature with JASCO DIP-360. ODS for column chromatography: YMC-Gel, ODS-AQ 120-S50; silica gel for tlc: Kiesel gel 60 F_{254} (Merck 1.05554).

Induction and subculture of the callus – The callus was induced from young leaves of the plant grown in the botanical garden, Bandung Institute of Technology, Indonesia. The medium for the induction and subculture was Murashige and Skoog (MS) medium containing 3% sucrose, 0.2% Gelrite (San-Ei Gen F.F.I.), 5 mM NAA, and 0.1 mM BA. The cultures were kept in the dark at 25℃ and subcultured at 3-week intervals.

Isolation and fractionation – Four weeks old callus (3.8 kg) was harvested and extracted at room temperature with 19 L of MeOH. The extract (120.0 g) was partitioned between ethyl acetate (1200 ml) and water (200 ml). The water soluble fraction was further extracted with 1-butanol. These 3 fractions were subjected to antimicrobial assay. The most active ethyl acetate soluble fraction (8.8 g) was chromatographed on silica gel (Wakogel C-100, 400 g) by stepwise elution with 800 ml each of the following solvents: hexane, hexane-EtOAc (95:5), hexane-EtOAc (90:10), hexane-EtOAc (80:20), hexane-EtOAc(60:40), hexane-EtOAc(40:60), EtOAc, EtOAc-MeOH (50:50) and MeOH. These 9 fractions (F1-F9) were subjected to antibacterial assay.

Antibacterial assay – Antibacterial assay was performed against *Escherichia coli* and *Bacillus subtilis*. The assay was conducted by twofold culture dilution method in a 96-well microplate.

Isolation of compounds 1 and 2 – The active fraction, F6 (230 mg), was subjected to ODS column chromatography (25 g in a steel column of ϕ 0.8×100 cm; flow rate, 1 ml/min) and eluted by H_2O-MeOH with increasing amounts of MeOH (60%-90%) to give 125 fractions of each 5 ml. The fractions indicating the similar pattern on silica gel tlc were combined. The most active combined fraction 73-79 (21.7 mg), was further chromatographed on an ODS column (3 g in a steel column of ϕ 0.4×50 cm; flow rate, 0.3 ml/min) by elution with H_2O-CH_3CN (4:6) to afford 200 fractions (0.3 ml/ fraction). The fractions exhibiting one spot on the tlc were combined to give pure compound 2 (5.2 mg). A less active but major constituent in the ethyl acetate fraction, compound 1, was isolated from the combined fraction 93-111 as colorless crystals (158 mg).

Compound **1**: colorless crystals, mp 293-295°; $[\alpha]_D$+2.6° (pyridine, *c* 1); EIMS: $[M]^+$ at *m/z* 456 ($C_{30}H_{48}O_3$); IR: 3423, 1726, 1675, 881

cm^{-1} ; ^1H NMR (DMSO-d_6): δ 0.63 (3H, s), 0.75 (3H, s), 0.86 (6H, s), 0.92 (3H, s), 1.63 (3H, s), 4.55 (1H, s), 4.67 (1H, s), 12.03 (1H, s); ^{13}C NMR (DMSO-d_6): δ 14.6, 15.9, 16.0, 16.1, 18.1, 19.1, 20.6, 25.2, 27.3, 28.3, 29.4, 30.3, 31.9, 34.1, 36.5, 36.9, 37.8, 38.4, 38.7, 40.4, 42.2, 46.8, 48.7, 50.1, 55.1, 55.6, 77.0, 109.8, 150.5, 177.4.

Compound **2**: colorless needles, mp 230-234°; $[\alpha]_D$+23.2° (pyridine, *c* 0.24); EIMS: *m/z* 472 [M^+] (3.7), 248 (100), 203 (50.0), 189 (18.1 %); HREIMS: *m/z* 472.3562, calculated for $C_{30}H_{48}O_4$ 472.3553; ^1H NMR (pyridine-d_5): δ 0.89 (3H, s), 0.95 (3H, s), 1.04 (3H, s), 1.11 (3H, s), 1.26 (3H, s), 0.92 (3H, d, *J*=6.7 Hz), 0.96 (3H, d, *J*=4.9 Hz), 2.60 (1H, d, *J*=10.9 Hz), 3.75 (1H, d, *J*=2.5 Hz), 4.28 (1H, ddd, *J* =11.6, 3.7, 3.0 Hz), 5.44 (1H, t, *J*=3.0 Hz); ^{13}C NMR (pyridine-d_5): δ 16.8, 17.48, 17.50, 21.4, 22.3, 22.7, 23. 9, 24.9, 28.7, 29. 5, 31.1, 33.2, 33.5, 37.5, 38.6, 38.8, 39.4, 39.5, 40.2, 42.6, 43.0, 47.9, 48.0, 48.7, 53.6, 66.1, 79.3, 125.6, 139.3, 179.9.

Methyl ester of compound **2**: colorless needles, mp 198-200°; $[\alpha]_D$+26.4° ($CHCl_3$, *c* 0.28); HREIMS: *m/z* 486.3711, calcd. for $C_{31}H_{50}O_4$ 486.3709; ^1H NMR ($CDCl_3$): δ 0.72 (3H, s), 0.84 (3H, s), 0.94 (3H, s), 1.00 (3H, s), 1.07 (3H, s), 3.58 (3H, s), 0.83 (3H, d, *J*=6.1 Hz), 0.92 (3H, d, *J*=6.1 Hz), 2.21 (1H, d, *J*=11.0 Hz), 3.41 (1H, s, br), 3.98 (1H, br.d, *J*=11.0 Hz), 5.23 (1H, t, *J*=3.6 Hz); ^{13}C NMR ($CDCl_3$): δ 16.4, 16.9, 17.0, 18.0, 21.2, 21.9, 23.3, 23.8, 24.2, 27.9, 28.5 30.7, 32.8, 36.6, 38.2, 38.3, 38.9, 39.0, 39.7, 41.9, 42.1, 47.2, 48.1, 48.1, 51.4, 53.0, 66.6, 79.0, 125. 5, 138.3, 178.0.

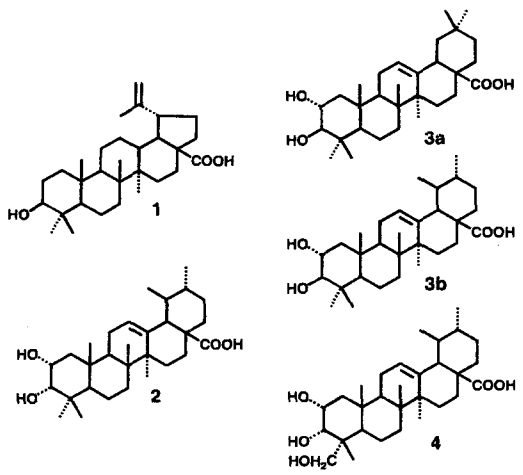
Isolation of substance 3 – An active fraction, F7 (370 mg), was chromatographed on an ODS column (25 g in a steel column of φ 0.8×100 cm; gradient elution with 60%-90% MeOH of a flow rate, 1 ml/min) and consecutively on a silica gel column (Wakogel C-300, 6 g in a steel column of φ 0.4×100 cm; gradient elution with 50%-70% EtOAc in hexane of a flow rate, 0.6 ml/min) to give substance 3 (16.1 mg) as colorless needles.

Substance **3**: colorless needles, mp 251-253°; $[\alpha]_D$+0.65° (pyridine, *c* 1); HREIMS: *m/z* 472.3565, calcd. for $C_{30}H_{48}O_4$ 472.3553); ^1H NMR (pyridine-d_5): δ 0.94 (3H, s), 0.98 (3H, s), 0.99 (3H, s), 1.02 (3H, s), 1.07 (3H, s), 1.26 (6H, s), 2.63 (1H, d, *J*=10.6 Hz), 3.30 (1H, dd, *J*=14.0, 4.3 Hz), 3.40(1H, dd, *J*=9.8, 3.1 Hz), 4.08 (1H, ddd, *J*=10.7, 10.1, 4.3 Hz), 5.46 (1H, dd, *J*=3.7, 3.0 Hz); ^{13}C NMR for **3a** (pyridine-d_5): δ 16.9, 17.5, 17.7, 18.9, 23.8, 23. 8, 24.0, 26.2, 28.3, 29.3, 31.0, 33.3, 33.3, 33.3, 34.3, 38.5, 39.85, 40.9, 42.3, 46.5, 46.7, 47.8, 48.1, 48.2, 55.9, 68. 6, 83.9, 122.4, 145.0, 180. 4; ^{13}C NMR for **3b** (pyridine-d_5): δ 17.0, 17.5, 17.6, 17.7, 18.8, 21.4, 23.7, 23.9, 24.9, 28.7, 29.3, 31.1, 33.5, 37.5, 38.4, 39.4, 39.5, 39.8, 40.0, 42.6, 48.0, 48.0, 48.1, 53.6, 55.9, 68.6, 83.8, 125.5, 139.4, 180.0.

Isolation of compound 4 – From a fraction, F8 (1.0 g), compound **4** (18.1 mg) was isolated by ODS and silica gel column chromatography in a similar way to the isolation of **3**.

Compound **4**: colorless needles, mp 216-219°; $[\alpha]_D$+2.2° (pyridine, *c* 1); HREIMS: *m/z* 488.3515, calcd. for $C_{30}H_{48}O_5$ 488.3502; ^1H NMR (pyridine-d_5): δ 0.86(3H, s), 0.99 (3H, s) 1.06 (3H, s), 1.14 (3H, s), 0.91 (3H, d, *J*=6.1 Hz), 0.95 (3H, d,*J*=6.7 Hz), 2.60 (1H, d, *J*=11. 6 Hz), 3.74 (1H, d, *J*=10.9 Hz), 3.91 (1H, d, *J*=10.9 Hz), 4.12 (1H, d, *J*=2.5 Hz), 4.26 (1H, ddd, *J*=11.0, 4.3, 3.0 Hz), 5.45 (1H, t, *J*=3.0 Hz); ^{13}C NMR (pyridine-d_5): δ 17.2, 17.5, 17.6, 17.8, 18.3, 21.4, 23.7, 23.9, 24.4, 28.7, 31.1, 33.2, 37.5, 38.4, 39.4, 39.5, 40.1, 41.9, 42.6, 42.8, 43.5, 48.0, 48.1, 53.6, 66.2, 71.3, 79.0, 125.5, 139.4, 179.9.

Quantitative analysis for compounds 1, 2, 3a, 3b and 4 – To perform the quantitative analysis of these triterpene acids, the acids were converted to benzoyl derivatives of their methyl esters. Commercially available authentic betulinic acid (**1**) as well as isolated triterpene acids, **2**, **3** (a mixture of **3a** and **3b**) and **4** were treated with excessive diazomethane. The methylated products were purified by silica gel column chromato-



graphy and treated with benzoyl chloride in pyridine to give the crude benzoates of the methyl esters **1, 2, 3** and **4**, which were further purified by a Sep-pak C18 cartridge (Waters Associates). Calibration curves were obtained using these standard samples. The standard solution of **3** exhibited two well separated peaks, the first eluting smaller (**3b**) and the second eluting larger (**3a**) peaks in a ratio of 49:51. The peak areas of injected standard solutions of 3 were plotted against the concentration (for **3a**, 51% of the original concentration of the standard solutions; for **3b**, 49% of that) to give calibration curves of **3a** and **3b**. Conditions of HPLC for quantitative analysis: Waters LC Module I equipped with U6K injector; column, ODS-3 (GL Sciences, ϕ 4.6×250 mm); mobile phase, H_2O-MeOH (8:92 to 1:99); flow rate 1 ml/min; detection, UV 234 nm.

Preparation of samples for quantitative analysis – The triterpene acid content of the callus and various parts of *T. grandis* was determined as follows. Four weeks old callus (5 g), the air dried leaves (2 g), bark (2 g) and wood (2 g) were extracted with MeOH. The extracts were then treated with diazomethane and subsequently with benzoyl chloride in pyridine. The reaction mixture was extracted with EtOAc, worked up in usual ways, and passed through a Sep-pak C_{18} cartridge prior to quantitative analysis.

Results and Discussion

Antibacterial activities of the compounds isolated – Antibacterial activities against *E. coli* and *B. subtilis* of compounds **1, 2, 3** and **4** are listed together with those of tetracycline and carbenicillin in Table 1. Substance **3** which was the most active among the isolated triterpenes was 10-30% as active as the two well known commercially available antibiotics.

Identification of compounds 1, 2, 3a, 3b and 4 – Compound **1**: Based on the ^1H and ^{13}C NMR spectral data, **1** was suggested to be a lupenic acid having one hydroxyl group. Comparison of the ^1H and ^{13}C NMR data of **1** with the published data of 3β-hydroxy-lup-20(29)-en-28-oic acid (betulinic acid) indicated an identity (Siddiqui *et al.*, 1988). Direct comparison of **1** with commercially available betulinic acid confirmed the identity in all respects. Therefore, **1** was identified as 3β-hydroxy-lup-20(29)-en-28-oic acid (1).

Compound **2**: Five tertiary methyls, two secondary methyls and one trisubstituted double bond implied by the NMR as well as the characteristic ^{13}C NMR chemical shift values of double bond carbons (δC 125.6 and 139.3) suggested that **2** had an ursene skeleton. The base peak at *m/z* 248 (observed: 248.1799, calculated: 248.1776 for $C_{16}H_{24}O_2$)

Table 1. Minimum inhibitory concentration of compounds **1, 2, 3** and **4** against *E. coli* and *B. subtilis*

Test organism	Compound	MIC (μg/ml)
E. coli	**1**	125
	2	31
	3	16
	4	125
	tetracycline	1.6
	carbenicillin	25
B. subtilis	**1**	62.5
	2	31
	3	16
	4	125
	tetracycline	6.3
	carbenicillin	3.1

of its EI-MS suggested that **2** was an ursen-28-oic acid and its two hydroxyl groups were located at the ring A or B (Manzoor and Habermehl, 1979). The ^{1}H and ^{13}C NMR data of **2** showed a close similarity to those of methyl 2α,3α-dihydroxy-urs-12-en-28-oate except the presence of ester methyl at C-28 (Kojima and Ogura, 1989). The ^{13}C and ^{1}H NMR of methyl ester of 2 showed quite an identity with the published data of methyl 2α,3α-dihydroxy-urs-12-en-28-oate (Kojima and Ogura, 1989). Thus, 2 was concluded to be 2α,3α-dihydroxy-urs-12-en-28-oic acid.

Substance **3**: Seven tertiary methyl groups, one trisubstituted double bond (δ_H 5.46, δ_C 122.4, 145.0), and two carbons bearing a hydroxyl group (δ_C 68.6 and 83.9) suggested that compound **3a** had an oleanene skeleton. Comparison of ^{1}H NMR signals and the major signals in the ^{13}C NMR spectrum with the published data of 2α,3β-dihydroxy-olean-12-en-28-oic acid (Ikuta *et al.*, 1995), indicated that compound **3a** was 2α,3β-dihydroxy-olean-12-en-28-oic acid. However, the ^{13}C NMR spectrum of **3** showed many minor signals besides the major signals assigned to 2α,3β-dihydroxy-olean-12-en-28-oic acid. The smaller signals at δ_C 125.5 and 139.4 suggested the presence of ursene skeleton. In addition, a smaller signal at δ_H 2.63 (d, J=10.6 Hz) was assignable to H-18 of ursene skeleton. Thus, substance **3** should be a mixture of acids (**3a** and **3b**) of oleanene and ursene skeleton, respectively. The minor signals in the ^{13}C NMR was identical with the published data of 2α,3β-dihydroxy-urs-12-en-28-oic acid (Kitajima and Tanaka, 1993). Therefore, the minor compound (**3b**) was identified as 2α,3β-dihydroxy-urs-12-en-28-oic acid. The HPLC analysis of the mixture **3** indicated the ratio of **3a** to **3b** was 51:49. Compound **3b** isolated from the wood which did not contain **3a** (Table 2) was as active as **3** (MIC against *E. coli* and *B. subtilis*: 16 μg/ml). This suggested that both **3a** and **3b** were equally active.

Table 2. Content of triterpene acids **1, 2, 3a, 3b** and **4** in the callus and various parts of the intact plant

Sample	Content (μg/g dry wt)				
	1	**2**	**3a**	**3b**	**4**
callus	387.6	70.8	23.6	20.0	58.5
leaves	150.5	ND[1]	1.8	1.7	ND[4]
wood	1117.2	ND[1]	ND[2]	141.6	ND[4]
bark	711.4	ND[1]	ND[2]	ND[3]	ND[4]

ND[1]: not detected, below a level of 23.6 μg/g dry wt.
ND[2]: not detected, below a level of 1.8 μg/g dry wt.
ND[3]: not detected, below a level of 1.7 μg/g dry wt.
ND[4]: not detected, below a level of 19.5 μg/g dry wt.

Compound **4**: Signals at δ_C 66.2, 71.3 and 79.0 were assigned to three carbons bearing a hydroxyl, and a signal at δ_C 179.9 to a carboxyl carbon. The ^{13}C NMR chemical shift values (δ_C 125.5 and 139.4) coupled with the presence of four tertiary methyls, two secondary methyls and one trisubstituted double bond suggested that compound **4** had an ursene skeleton. Compound **4** was identified with 2α,3α, 23-trihydroxy-urs-12-en-28-oic acid in all respects (Sashida *et al.*, 1992).

Triterpene acid content of callus and various parts of *T. grandis* – The content of triterpene acids **1, 2, 3a, 3b** and **4** in the callus and various parts of the plant were shown in Table 2. Compound **1** was present in the aerial parts of the plant and also in the cultured callus. Compounds **2** and **4** were detected in the cultured callus, whereas they were not detected in the aerial parts of the plant. Compounds **3a** and **3b** were detected in the callus ca. 10 times as much as in the leaves. This result suggests the possibility of producing these antibacterial compounds by cell culture.

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