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Cytotoxic and antimalarial constituents from the roots of *Eurycoma longifolia*

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Abstract—Sixty-five compounds were isolated from the roots of *Eurycoma longifolia* and characterized by comprehensive analyses of their 1D and 2D NMR, and mass spectral data. Among these isolates, four quassinoid diterpenoids were reported from natural sources for the first time, namely eurycomalide A (**1**), eurycomalide B (**2**), 13β, 21-dihydroxyeurycomanol (**3**), and 5α, 14β, 15β-trihydroxyklaineanone (**4**). Screening of cytotoxicity, anti-HIV and antimalarial activity of these isolated compounds was also furnished by in vitro assays. Compounds **12**, **13**, **17**, **18**, **36**, **38**, **59**, and **62** demonstrated strong cytotoxicity toward human lung cancer (A-549) cell lines, however, **12**, **13**, **17**, **38**, **57**, **58**, and **59** exhibited strong cytoxicity toward human breast cancer (MCF-7) cell lines. Compounds **57** and **58** displayed potent antimalarial activity against the resistant *Plasmodium falciparum*. The thorough studies on the stereochemistry of the different quassinoid diterpenoids provide a clear reference to the scientists who are interested on this field.

1. Introduction

Eurycoma longifolia, native to Burma, Indochina, Thailand, and Southeast Asia is a tall Simaroubaceous slender shrub-tree commonly found as an understroy in the lowland forests at up to 500 m above sealevel. *E. longifolia* known locally as 'Tongkat Ali' in Malaysia, 'Pasakbumi' in Indonesia, 'Cay ba binh' in Vietnam and 'Ian-don' in Thailand is popularly sought after in herbal remedies and has been frequently prescribed either as a single ingredient or as a mixture with other herbs. The roots of this plant are used as folk medicine for the treatment of aches, persistent fever, tertian malaria, sexual insufficiency,[1] dysentery, glandular swelling,[2-3] and as health supplements.[1] Recently, Lin et al. reported that the crude ethanolic extract of the root of *E. longifolia* could decrease the basal release of testosterone but increase the human chorionic gonadotropin (hcG)-induced production of testosterone by rat leydig cells.[4] From the roots, several classes of compounds have been identified and they included quassinoids,[5-8] canthin-6-one alkaloids,[5] β-carboline alkaloids,[5] tirucallane-type triterpenes,[8] squalene derivatives,[9] and biphenylneolignans.[10] Some of these constituents were shown to possess cytotoxic,[5, 8, 11] antimalarial,[12] anti-ulcer,[13] antipyretic,[14] and plant growth inhibition activities.[15] In addition, the crude extracts of this plant were reputed to increase male virility and sexual prowess and gained notoriety as a male aphrodisiac.[16–18] The wide spectrum of pharmacological activities associated with the constituents and crude extracts of the title plant has further prompted us to undertaken the chemical investigation of the methanolic extract of the root of *E. longifolia*. In a previous paper, we reported the isolation and structural elucidation of cytotoxic canthin-6-one and β-carboline alkaloids.[19] As a result of further fractionation efforts, two new C_{19}-skeleton quassinoids (**1**,**2**), two new highly oxygenated klaineanone type C_{20} quassinoids (**3**,**4**), and sixty-one known compounds were isolated from the root of *E. longifolia*. Hence, we wish to report on the isolation and characterization of compounds (**1**–**4**) (Fig. 1) and the cytotoxicity, antimalarial and anti-HIV activities.

2. Results and discussion

Eurycoma longifolia Jack roots were procured from Malaysia and were extracted with methanol under reflux. The methanol extract was partitioned into chloroform, *n*-butanol, and water and the chloroform

Keywords: Quassinoid diterpenoids; Cytotoxcity; Antimalarial activity; Spectroscopic characteristics.
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soluble portion was subjected to repeated column chromatography (CC) over silica gel and preparative thin layer chromatography (PTLC) to afford two new quassinoids **1**–**2**, along with 41 known compounds **6**–**46**. The *n*-butanol and water extracts were chromatographed separately on Diaion HP-20 and the fractions obtained were further purified by conventional isolation procedures to give two new quassinoid derivatives **3**–**4** and 20 known constituents **5** and **47**–**65**.

Compound **1** was obtained as optically active colorless needles with mp 178–180° and $[\alpha]_D^{25}$ +31.6°. The molecular formula $C_{19}H_{26}O_6$ established from its pseudomolecular ion peak at *m*/*z* 351.1806 in HRFABMS was shown to have C_{19} quassinoid skeleton, and was further corroborated by ^{13}C NMR spectrum which showed signals for all the 19 carbons of the molecule. DEPT experiments indicated the presence of four methyls, one methylene, nine methines, as well as five quaternary carbons in the molecule. The UV spectrum of **1** exhibited strong absorption maxima at 234 and 283 nm compatible with an α, β-unsaturated carbonyl chromophore. The IR absorption bands at 3423, 1754 and 1657 cm^{-1} were in agreement with the presence of hydroxyl, lactonic carbonyl, and α, β-unsaturated carbonyl functionalities, respectively. The ^1H NMR spectrum showed two secondary methyls at δ 0.94 (3H, d, J = 6.4 Hz, CH_3-16) and 1.05 (3H, d, J = 6.9 Hz, CH_3-15), and two tertiary methyls at δ 1.68 (3H, s, CH_3-18) and 1.92 (3H, s, CH_3-17). In the ^1H-^1H COSY spectrum, the H-1 (δ 3.56) and H-2 protons (δ 4.14) attached to hydroxyl bearing carbons were coupled mutually. The latter proton showed further coupling with the C-3 methylene protons (δ 1.23 and 2.13), and in turn these methylene protons coupled with H-4 (δ 2.53), provided strong support for the formulation of the ring A structure. A strong UV absorption at 234 nm due to α, β-unsaturated ketone together with ^1H and ^{13}C NMR signals at δ 5.92 (120.5) and δ 3.38 (53.0) characteristic of a conjugated olefinic proton and juncture were consistent with the B ring structure. In addition, two methylene protons at δ 1.23 (1H, dt, J = 12.5, 12.3 Hz, H-3a) and 2.13 (1H, dt, J = 12.3, 4.5 Hz, H-3e) which showed ^2J, ^3J-HMBC correlations with carbons at δ 32.2 (C-4), 70.4 (C-2), 85.2 (C-1), and 171.0 (C-5) suggested the partial structure of A- and B-rings, which was also supported by the HMBC correlations (Fig. 2) between olefinic hydrogen at δ 5.92 (1H, s, H-6) and δ 32.2 (C-4), 46.5 (C-10), and 47.8 (C-8), respectively. From the COSY spectral analysis, proton signals at δ 3.38 (1H, br s, H-9), 4.49 (1H, d, J = 4.9 Hz, H-11), 5.78 (1H, ddd, J = 6.0, 4.9, 3.2 Hz, H-12), 2.46 (1H, d, J = 3.2 Hz, H-13), and one D_2O exchangeable doublet at δ 6.91 (1H, J = 6.0 Hz, OH-12), revealed the coupling network around a rarely-occurred C-ring, the OH-11 of which was lactonized with the carbonyl group at C-13. Further evidence of this unique arrangement came from the-$CHCH_3$ fragment at δ 3.31 (1H, q, J = 6.9 Hz, H-14) and 1.05 (3H, d, J = 6.9 Hz, CH_3-15), and HMBC correlations from H-14 to C-8 and C-19, and H-9 to C-1 and C-7. The stereochemistry of this compound was confirmed through the NOESY experiments. Observation of NOESY correlations among CH_3-17, H-2, and H-4; among H-11, H-12, H-13, and H-14; and absence of NOE between H-1 and H-9, suggested that CH_3-16, OH-2, and OH-12 groups were both in α-configurations and that the B/C ring juncture was in a *cis*-relationship. By contrast, the stereochemistry of OH-1 group was assigned to β-configuration by observing the NOESY correlation between H-1 and H-3a. From these findings, the structure of eurycomalide A was proposed to be **1**.

Eurycomalide B (**2**), obtained as optical active colorless syrup, $[\alpha]_D^{25}$ −25.0°, possessed the eurycomalactone-type C_{19}-quassnoid skeleton, $C_{19}H_{24}O_6$, which was confirmed by HRFABMS. The UV spectrum of **2** exhibited strong absorption maxima at 283 nm according to an α, β-unsaturated carbonyl moiety. The IR absorption bands at 3424, 1743, and 1658 cm^{-1} also revealed the presence of hydroxyl, lactonic carbonyl, and α, β-unsaturated carbonyl functionalities, respectively. Typical ^1H NMR signals, including a vinyl methyl at δ 1.79 (3H, s, CH_3-16), two tertiary methyls at δ 1.77 (3H, s, CH_3-18) and 1.89 (3H, s, CH_3-17), and a secondary methyl doublet at δ 1.05 (3H, d, J = 7.0 Hz, CH_3-19), and nineteen carbon signals containing the lactonic carbon signal at δ 177.3 in its ^{13}C NMR spectrum were also found to support the eurycomalactone quassinoids basic skeleton. Except for the four methyl signals in the ^1H NMR spectrum, three methines at δ 2.60 (1H, d, J = 3.1 Hz, H-9), 3.33 (1H, m, H-13), 3.41 (1H, s, H-14); four oxygenated methines at δ 3.94 (1H, d, J = 7.5 Hz, H-1), 4.50 (1H, m, H-12), 4.70 (1H, d, J = 7.5 Hz, H-2), and 5.99 (1H, m, H-11); and two olefinic protons at δ 6.08 (1H, s, H-6) and 6.21 (1H, s, H-3), inferred that the coupling sequence of A, B, and C rings was similar to that of eurycomalactone (**12**).[20] In the ^1H–^1H COSY spectrum, the H-2 resonance at δ 4.70 correlated to H-1 and H-3, H-3 correlated to a vinyl methyl (CH_3-16); in the HMBC experiment, the ^2J, ^3J-correlations between CH_3-16 and C-5, and H-6 and C-5, inferred the presence of a fragment of CHOH-CH=C(CH_3)-C= CH-CO which was commonly occurred in the eurycomalactone-type quassinoids with carbonyl as C-2. However, the detailed HMBC analysis showed that the carbonyl at δ 199.5 (C-7) exhibited ^3J-correlation with CH_3-18, and the H-1 which has coupled with H-2 in the ^1H NMR and COSY spectra also displayed ^2J, ^3J-HMBC relationships to C-10 and CH_3-17. It suggested that the fragment was arranged in different pattern with carbonyl as C-7 (Fig. 1). The complete proton and carbon assignments were furnished by a combination of COSY, NOESY, HMQC, and HMBC experiments. The NOE correlations which were drawn in Figure 3 determined the stereochemistry of **2** similar to that of eurycomalactone. The OH-2 α-orientation was deduced from the NOE correlation between H-2 and CH_3-17. From the above spectral analyses, the structure of eurycomalide B was established as **2** shown in Figure 1.

13β, 21-Dihydroxyeurycomanol (**3**) was obtained as colorless amorphous powder with optical activity, $[\alpha]_D^{25}$ +51.4°, and determined as $C_{20}H_{28}O_{11}$ from the pseudomolecular ion peak $[M+Na]^+$ at *m*/*z* 467.1526 in its HRFABMS. The IR absorption band at 1735 cm^{-1} was indicative of a lactonic carbonyl functionality. Two

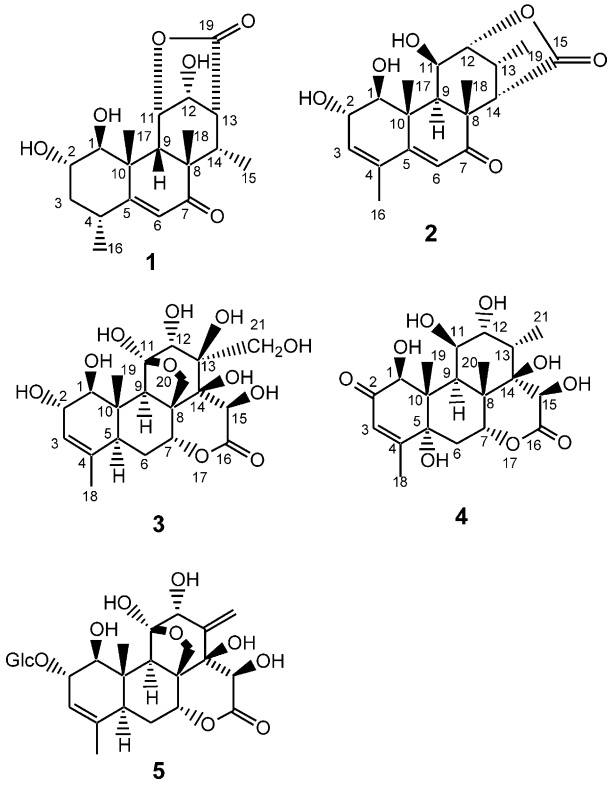

Figure 1. Structures of isolated compound **1**–**5**.

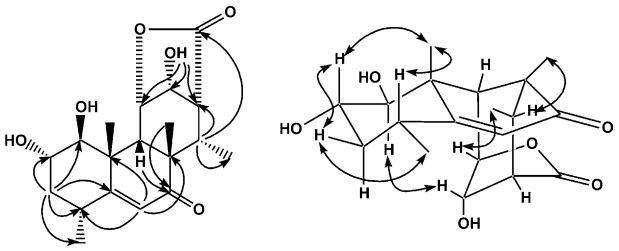

Figure 2. Key HMBC and NOESY Correlations of **1**.

singlet methyls were located at δ 1.60 (3H, CH_3-18) and 1.76 (3H, CH_3-19) in its ^{1}H NMR spectrum. The ^{13}C NMR spectrum revealed the prensence of 20 carbons, including a lactone carbonyl, a hemiketal carbon, a methyl, a vinyl methyl, and two olefinic carbons, indicative of C_{20} quassinoid skeleton. Observation of the C-2 resonance at δ 72.7 as a methine carbon, the ^{1}H-^{1}H COSY of H-2 (δ 4.60) with H-1 and H-3, the long range COSY of H-3 with H-5 and a vinyl methyl (CH_3-18), and absence of an UV absorption of α, β-unsaturated ketone suggested the A-ring structure. On the other hand, the ^{1}H and ^{13}C NMR spectral data of **3** corresponded to those of a known C_{20} quassinoid, eurycomanol,[21] except for the substituents on the C-ring system, that is, the presence of a hydroxyl and hydroxymethyl groups in place of a terminal methylene at C-13 of eurycomanol was suggested by the ^{13}C signals at δ 78.0 and 67.6 and the ^{1}H signals at δ 5.00 (1H) and 4.60 (1H), assignable to C-13 and C-21 hydroxymethyl group. The stereostructure of **3** was deduced from the NOESY correlations showed in Figure 4 and from coupling constants. The NOESY crosspeaks among H-1, H-5, and H-9 inferred the β-configuration for C-1

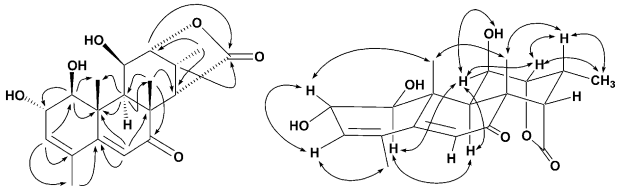

Figure 3. Important HMBC and NOESY correlations of **2**.

hydroxyl group. The coupling constant (8.4 Hz) of H-1 and no NOE with H-2 established the α-configuration for C-2 hydroxyl group. The NOESY correlations between H-19 and H-20, H-12 and H-21, H-9 and H-15, suggested the α-configurations for hydroxyl groups at C-11 and C-12, and β- for hydroxyl groups at C-13, C-14 and C-15, respectively. Therefore, the structure of **3** was elucidated as shown.

5α, 14β, 15β-Trihydroxyklaineanone (**4**) obtained as optically active colorless syrup, $[\alpha]_D^{25}$ +31.8°, was shown to have the klaineanone type C_{20} skeleton, $C_{20}H_{28}O_9$, by the pseudomolecular ion peak at *m*/*z* 413.1808 in the HRFABMS. The UV spectrum showed absorption maxima at 227 nm due to an α, β-unsaturated carbonyl chromophore. The IR absorption bands at 3380, 1739, and 1655 cm^{-1} indicated the presence of hydroxyl, lactonic carbonyl, and α, β-unsaturated carbonyl functionalities, respectively. The ^{1}H and ^{13}C NMR data of **4** suggested that the C-11, 20 hemiketal arrangement was not present and the spectroscopic data of B, C, and D rings were almost identical with those of 14β, 15β-dihydroxyklaineanone (**38**).[22] In ^{1}H NMR spectrum, the chemical shifts of H-1 and H-3 were also similar to those of **38**; however, H-5 signal was not observed. The absence of proton signal for H-5 in ^{1}H NMR spectrum and occurrence of one more oxygenated quaternary carbon in ^{13}C NMR spectrum implied that C-5 was oxidized to tertiary alcohol, which was also inferred by the ^3J HMBC correlations (Fig. 5) of a quaternary carbon signal at δ 75.4 with proton signals at δ 1.75 (3H, s, CH_3-19), 1.98 (3H, s, CH_3-18), and 6.18 (1H, s, H-3). The stereostructure was corroborated by the NOE relationships observed in the phase-sensitive NOESY spectrum. The A/B ring *trans* juncture and thus configuration of hydroxyl at C-5 can be interpreted to be α by the NOESY correlations of H-9 with H-1 and H-11, and CH_3-19 with CH_3-20 and H-6. Using a combination of homo and hetero nuclear two-dimensional NMR techniques (^{1}H-^{1}H COSY, NOESY, HMQC, and HMBC), complete assignment of the ^{1}H and ^{13}C signals of **4** were successfully performed. These observations along with the molecular formula demonstrated by $C_{20}H_{28}O_9$ have led to the conclusion that 5α, 14β, 15β-trihydroxyklaineanone has structure **4**.

Eurycomanol-2-*O*-β-D-glucopyranoside (**5**), isolated as colorless needles, mp 228–230 and $[\alpha]_D^{25}$ +74.50°, with the molecular formula $C_{26}H_{36}O_{14}$, which was reported in earlier communication.[23] The ^{13}C NMR spectrum revealed the presence of one β-glucopyranosyl unit together with 20 carbon signals for the aglycone, which suggested a C_{20} quassinoid skeleton. DEPT experiment indicated the presence of two methyls, three methylenes,

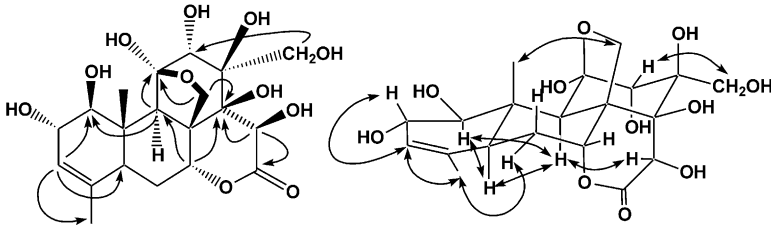

Figure 4. Key HMBC and NOESY correlations of **3**.

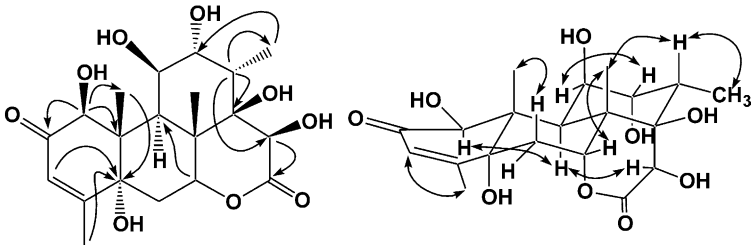

Figure 5. Key HMBC and NOESY correlations of **4**.

eight methines, as well as seven quaternary carbons in the aglycone moiety. Comparison of the ^{1}H and ^{13}C NMR spectral data of **6** with those reported revealed that the values for H-1 (C-1), H-2 (C-2), H-7 (C-7), and H-15 (C-15) were assigned reversely in the literature. Therefore, the complete assignments were established by using ^{1}H-^{1}H COSY, NOESY, HMQC, and HMBC experiments and coupling constants in the ^{1}H NMR spectrum. A proton signal at δ 4.58 (1H, m) exhibited ^{1}H-^{1}H COSY with δ 5.93 (1H, br s, H-3) was attributed to H-2, and it connected to δ 84.1 in the HMQC spectrum, which therefore assigned as C-2. In contrast, carbon signal at δ 82.6 displsyed HMBC correlation with δ 1.58 (3H, s, CH_3-19) was assigned as C-1. In addition, a singlet at δ 5.38 showed ^{2}J HMBC correlation with δ 173.8 (C-16) was ascribed to H-15, and it exhibited C-H one-bond coupling (^{1}J) with δ 71.8 (C-15); one broad singlet at δ 5.11 attributable to H-7 was correlated with signals at δ 1.86 (1H, dd, $J=11.1$, 9.8 Hz, H-6β) and 2.10 (1H, br d, $J=11.1$ Hz, H-6α) in ^{1}H-^{1}H COSY spectrum and connected to a carbon at δ 76.2 in HMQC spectrum.

In addition, each of known compound **6-65** isolated from *E. longifolia* was identified as mixture of β-sitosterol (**6**) and stigmasterol (**7**),[24] scopoletin (**8**),[24] *p*-hydroxybenzaldehyde (**9**),[25] methyl β-carboline 1-carboxylate (**10**),[19] 4, 5-dimethoxycanthin-6-one (**11**),[19] eurycomalactone (**12**),[20] 6-dehydroxylongilactone (**13**),[26] laurycolactone B (**14**),[20] 6-hydroxy-5, 6-dehydroeurycomalactone (**15**),[20] syringic aldehyde (**16**),[25] 9-methoxycanthin-6-one (**17**),[19] canthin-6-one (**18**),[19] eurylene (**19**),[27] 2, 4′-dihydroxy-3′-methoxyacetophenone (**20**),[28] laurycolactone A (**21**),[20] 6α-hydroxyeurycomalactone (**22**),[22] 10-methoxycanthin-6-one (**23**),[19] 8-hydroxy-9-methoxycanthin-6-one (**24**),[19] 9,10-dimethoxycanthin-6-one (**25**),[19] 5-methoxycanthin-6-one (**26**),[19] fraxidin (**27**),[29] *n*-pentyl β-carboline-1-propionate (**28**),[19] 3-hydroxy-1-(4′-hydroxy-3′-methoxyphenyl)propan-1-one (**29**),[30] lariciresinol (**30**),[31] 9-hydroxycanthin-6-one (**31**),[19] 5-hydroxymethylcanthin-6-one (**32**),[19] canthin-6-one 3*N*-oxide (**33**),[19] 5-hydroxymethyl-9-methoxycanthin-6-one (**34**),[19] 1-hydroxy-9-methoxycanthin-6-one (**35**),[19] longilactone (**36**),[22] 9-methoxycanthin-6-one 3*N*-oxide (**37**),[19] 14,15β-dihydroxyklaineanone (**38**),[22] 5,6-dehydroeurycomalactone (**39**),[20] vanillic acid (**40**),[25] protocatechuic acid (**41**),[32] syringic acid (**42**),[33] β-sitosteryl glucoside (**43**),[33] 4-hydroxy-5-methoxycanthin-6-one (**44**),[19] β-carboline-1-propionic acid (**45**),[19] 7-methoxy-β-carboline-1-propionic acid (**46**),[19] sodium syringate (**47**),[34] sodium *p*-hydroxybenzoate (**48**),[35] nicotinic aicd (**49**),[33] adenosine (**50**),[36] guanosine (**51**),[37] thymidine (**52**),[38] *erythro*-1-*C*-syringylglycerol (**53**),[36] *threo*-1-*C*-syringylglycerol (**54**),[36] *erythro*-guaiacylglycerol (**55**),[39] *threo*-guaiacylglycerol (**56**),[39] eurycomanone (**57**),[13] pasakbumin B (**58**),[13] pasakbumin C (**59**),[13] iandonone (**60**),[40] *threo*-1,2-bis-(4-hydroxy- 3-methoxyphenyl)propane-1, 3-diol (**61**),[41] canthin-6-one 9-*O*-β-glucopyranoside (**62**),[19] 9-hydroxycanthin-6-one 3*N*-oxide (**63**),[19] picrasidine L (**64**),[19] and 1-hydroxycanthin-6-one (**65**)[19] by comparison of their physical and spectral data with those reported in the literature.

Compounds **3**, **12**, **13**, **17**, **18**, **19**, **31**, **36**, **37**, **38**, **44**, **45**, **46**, **57**, **58**, **59**, and **62** were screened for in vitro cytotoxicity against A-549 and MCF-7 tumor cell lines[42] and inhibition of HIV replication in H9 lymphocytes except for **19** and **58**.[43] Compounds **12**, **13**, **17**, **18**, **36**, **38**, **59**, and **62** demonstrated strong cytotoxicity toward A-549 cell lines, however, **12**, **13**, **17**, **38**, **57**, **58**, and **59** displayed strong cytoxicity toward MCF-7 cell line (Table 1). None of the tested compounds showed significant anti-HIV effects. Compounds **3**, **5**, **10**, **12**, **13**, **14**, **17**, **18**, **28**, **31**, **37**, **38**, **44**, **45**, **46**, **57**, **58**, **59**, **62**, and **63** were evaluated for antimalarial activity against two *Plasmodium falciparum* clones, W2 and D6.[44–46] Compounds **57** and **58** exhibited strong antimalarial activity against both the W2 and D6 *P. falciparum* clones and compounds **12**, **38**, and **59** showed weak activity against both the W2 and D6 clones (Table 2).

Table 1. Cytotoxicity against human cancer cell lines, A-549 and MCF-7

Sample	ED_{50}[a]	
	A-549	MCF-7
3	>20 (13)	>20 (11)
12	<2.5 (74)	<2.5 (72)
13	<2.5 (70)	<2.5 (64)
17	<2.5 (55)	4.5
18	3.6	7.3
19	>20 (22)	>20 (28)
31	10.0	19.6
36	4.6	6.1
37	18.5	18.9
38	<2.5 (68)	<2.5 (69)
44	16.2	18.1
45	NA	>20 (8)
46	>20 (7)	>20 (15)
57	8.1	<2.5 (55)
58	8.4	<2.5 (55)
59	3.6	<2.5 (55)
62	4.2	16.1

[a] ED_{50} in μg/mL. If inhibition <50% at 20 μg/mL or >50% at 2.5 μg/mL then percent observed is the value in bracket.

Table 2. Antimalarial activity against the resistant *Plasmodium falciparum*

Target/sample	IC_{50} (ng/mL)	
	W2	D6
Mefloquine	1.013	3.210
Chloroquine	75.270	2.722
3	1282.703	1281.629
5	—	—
10	—	—
12	167.628	296.089
13	568.908	885.394
14	—	—
17	—	—
18	2238.471	—
28	—	—
31	2335.894	—
37	—	—
38	147.064	210.532
44	3525.352	2957.416
45	—	—
46	—	—
57	14.912	26.094
58	22.658	34.001
59	93.389	158.601
62	—	2903.380
63	—	—

3. Conclusion

The present investigation on the roots of *E. longifolia* resulted in the isolation and characterization of a novel quassinoid, eurycomalide A (**1**), a eurycomalactone type quassinoid, eurycomalide B (**2**), and two klaineanone type quassinoids, 13β, 21-dihydroxyeurycomanol (**3**) and 5α, 14β, 15β-trihydroxyklaineanone (**4**), along with 61 known compounds of several classes. In the screening of cytotoxicity, eurycomalactone (**12**), 6-dehydroxylongilactone (**13**), 9-methoxycanthin-6-one (**17**), 14, 15β-dihydroxyklaineanone (**38**), and pasakbumin C (**59**) demonstrated strong cytotoxicity toward A-549 and MCF-7 cell lines, whereas canthin-6-one (**18**), longilactone (**36**), and canthin-6-one 9-*O*-β-glucopyranoside (**62**); and eurycomanone (**57**), and pasakbumin B (**58**) displayed strong cytotoxicity toward A-549 and MCF-7 cell lines, respectively. Eurycomanone (**57**) and pasakbumin B (**58**) exhibited marginal antimalarial activity against both the W2 and D6 *P. falciparum* clones. As we have isolated totally 19 quassinoids of four types, eurycomalactone, laurycolactone, klaineanone, and longilactone in this study, we have studied the spectroscopic characteristics of quassinoid diterpenes clearly. From the chemical shift of secondary methyl group in ^{1}H NMR spectrum, the basic skeleton of diterpenoids (Fig. 6) can be identified in the early stage. In case of eurycomalactone and laurycolactone type quassinoids, it falls in the range of δ 0.82–1.19, and appears in the most upfield region among the four methyl groups. However, for klaineanone and longilactone types, it resonates at δ 1.65–1.88 and appears between the primary methyl signals. In the extensive literature survey, there were no reports of anthraquinones from this plant except the report by Lin et al.[4] In our thorough examination of the roots of *E. longifolia*, we have also not found any anthraquinones. As Lin et al. separated similar anthraquinones from *Ventilago leiocarpa*[47] by using the Diaion HP-20 gel and reused the gel for isolation of the constituents from *E. longifolia*, it was assumed that the reported anthraquinones were contaminated from Diaion HP-20 gel.

4. Experimental

Melting points were determined on Yanaco MP-S3 micro-melting point apparatus without correction. Optical rotations were measured on a Jasco DIP-370 polarimeter. UV spectra were taken on a Hitachi UV-3210 spectrophotometer. IR spectra were recorded on a JASCO IR Report-100 spectrophotometer as KBr discs. ^{1}H, ^{13}C, COSY, HMQC, HMBC, and NOESY NMR spectra were recorded on the Bruker Avance-300 and AMX-400 NMR spectrometers, using tetramethylsilane (TMS) as the internal standard, and all chemical shifts were reported in parts per million (ppm, δ). High performance liquid chromatography (HPLC) was performed on a Shimadzu LC-10AT*VP* series pumping system equipped with a Shimadzu SPD-M10A*VP* diode array detector, a Merck Lichrospher® 100 RP-18 (5μm) column, and a Rheodyne injector. Column chromatography was carried out with silica gel [Kieselgel 60, 70–230 and 230–400 mesh (Merck)]. TLC was conducted on 0.25 mm precoated silica gel plates (60 F_{254}, Merck). All the mass and high-resolution mass spectra (FAB) were obtained on a Jeol JMS-700 spectrometer. CD spectra were recorded with a Jasco J-720 spectropolarimeter.

4.1. Plant material

The dried roots of *E. longifolia* Jack were collected from Malaysia in January, 2001, and authenticated by Prof. J. B. Wu, Graduate Institute of Pharmaceutical Chemistry, China Medical University, Taichung. A voucher specimen (TSWu 20010005) has been deposited at the Herbarium of National Cheng Kung University, Tainan, Taiwan.

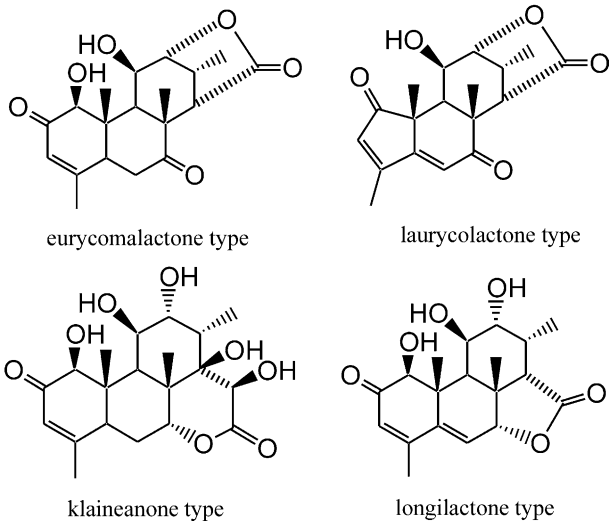

Figure 6. Basic skeletons of diterpenoids.

4.2. Extraction and isolation

The dried roots (10.5 kg) were cut into small pieces, extracted with methanol (20 L×7) under reflux, and concentrated to give dark brown syrup (500 g). The crude extract was partitioned with chloroform and *n*-butanol, successively, to afford four individual portions, chloroform layer, *n*-butanol layer, water layer, and residue. The chloroform layer was concentrated *in* vacuo to leave brown syrup (50 g). The residue was chromatographed on silica gel column and eluted with gradients of chloroform and acetone to give 12 fractions. Mixture of **6** and **7** (125.0 mg) was crystallized from the first fraction. Fraction 2 was rechromatographed with silica gel column using chloroform–methanol (99:1) as eluents and further recrystallization of the subfractions afforded **8** (40.0 mg), **9** (1.0 mg), **10** (2.0 mg), and **11** (1.2 mg). Fraction 3 was subjected to chromatography on a silica gel column with mixture of *n*-hexane–acetone (3:1) to obtain **12** (95.0 mg), **13** (8.0 mg), **14** (1.5 mg), **15** (1.0 mg), and **16** (3.5 mg). Fraction 4 was repeatedly column chromatographed over silica gel and eluted with *n*-hexane–ethyl acetate (2:1) to yield **17** (125.5 mg), **18** (180.0 mg), **19** (9.5 mg), **20** (2.5 mg), **21** (2.0 mg), and **22** (1.0 mg). Fraction 5 was separated by column chromatography on a silica gel column and eluted with a gradient of *n*-hexane and ethyl acetate to afford **23** (1.0 mg), **24** (2.5 mg), **25** (2.0 mg), **26** (1.5 mg), and **27** (2.2 mg), successively. Silica gel column chromatography of fraction 6 and further purification with PTLC with mixture of *n*-hexane and ethyl acetate (2:1) resulted in **28** (3.3 mg) and **29** (1.8 mg). Recrystallization of seventh fraction with benzene afforded **30** (3.2 mg). Fraction 8 was also subjected to column chromatography over silica gel with diisopropyl ether–methanol (9:1) and afforded **31** (90.8 mg), **32** (1.5 mg) and **33** (1.2 mg). Silica gel column chromatography of fraction 9 by *n*-hexane with a step gradient of ethyl acetate gave four subfractions. Subfraction 9-1 was further chromatographed on a silica gel column and eluted with *n*-hexane and ethyl acetate (2:1) to yield **34** (3.5 mg), **35** (3.8 mg) and **36** (25.0 mg). Subfraction 9-2 was subjected to silica gel column chromatography with diisopropyl ether–methanol (99:1) and further recrystallization of each subfractions afforded **1** (0.9 mg), **2** (0.4 mg), **37** (10.2 mg), and **38** (5.5 mg). Silica gel column chromatography on subfraction 9-3 with mixing eluents of benzene–acetone (1:1) resulted in **39** (3.5 mg), **40** (10.5 mg), **41** (3.5 mg), and **42** (8.8 mg). Compound **43** (20.5 mg) was crystallized from fraction 10 after recrystallization with methanol. Fraction 11 on C-18 gel column chromatography gave five subfractions and further PTLC on subfraction 11-3 yielded **44** (1.0 mg); in addition, recrystallization of subfraction 11-2 and 11-4 afforded **45** (10.3 mg) and **46** (10.5 mg), respectively.

The *n*-butanol layer (200 g) and residue (30 g) were combined and subjected to Diaion HP-20 column chromatography, which were eluted with a step gradient of water and methanol to give 6 fractions. Fraction 2 on column chromatography with silica gel by mixture of ethyl acetate–methanol (9:1) saturated with water gave 8 subfractions, and followed recrystallization of subfractions 2-2, 2-3, 2-6, 2-7, 2-8 yielded **47** (2.5 mg), **48** (1.2 mg), **49** (3.8 mg), **50** (10.8 mg) and **51** (6.8 mg), respectively. Subfraction 2-4 was further purified by HPLC with methanol–water (15:85) to give **52** (2.2 mg). Similar conditions on subfractions 2-5 and 2-6 afforded **53** (2.6 mg) and **54** (1.8 mg); **55** (1.8 mg), and **56** (1.0 mg), respectively. Fraction 3 was repeatedly column chromatographed on silica gel with solvent pair of chloroform–methanol (9:1) saturated with water to afford 10 subfractions. Subfractions 3-2 was further purified on silica gel column chromatography with mixture of chloroform-methanol–water (9:1:sat.) to yield **57** (220.8 mg). Similar conditions on subfraction 3-5 and 3-7 resulted in **5** (40.1 mg) and **58** (120.4 mg); and **59** (5.7 mg) and **60** (50.1 mg), respectively. Further separation procedures furnished by HPLC with mixing eluents of acetonitrile and water (25:75) on subfraction 3-4 afforded **3** (10.5 mg), **4** (0.4 mg), and **61** (3.3 mg). Silica gel column chromatography on fraction 4 eluted by chloroform–methanol (9:1) saturated with water divided it into 6 subfractions. Subfractions 4-1, 4-2, 4-3, and 4-4 was subjected to silica gel column chromatography with mixing solvent of chloroform–methanol (9:1) saturated by water to give **62** (20.5 mg), **63** (6.0 mg), and **64** (1.2 mg), respectively. Purification of fraction 5 on silica gel column chromatography with chloroform–methanol–water (9:1:sat) solvent pair resulted in the isolate **65** (1.5 mg).

The water layer (220 g) was directly chromatographed on Diaion HP-20 column and eluted with a gradient of water and methanol to give 5 fractions. Further purification on silica gel column chromatography with mixture of chloroform–methanol–water (9:1:sat.) on fractions 2, 3, and 4 yielded **57** (35.0 mg), **58** (15.0 mg), and **5** (10.8 mg), respectively.

4.2.1. Eurycomalide A (1). Colorless needles (MeOH), mp 178–180 °C; $[\alpha]_D^{25}$ +31.6° (*c* 0.086, MeOH); UV (MeOH) λ_{max} (log ε) 234 (4.09), 283 (3.04) nm; IR (KBr) ν_{max} 3423, 2932, 1754, 1657, 1270 cm^{-1}; ^1H

NMR (Pyridine-d_5, 300 MHz), δ 0.94 (1H, d, $J=6.4$ Hz, CH_3-16), 1.05 (1H, d, $J=6.9$ Hz, CH_3-15), 1.23 (1H, dt, $J=12.5$, 12.3 Hz, H-3a), 1.68 (3H, s, CH_3-18), 1.92 (3H, s, CH_3-17), 2.13 (1H, dt, $J=12.3$, 4.5 Hz, H-3e), 2.46 (1H, d, $J=3.2$ Hz, H-13), 2.52 (1H, m, H-4), 3.31 (1H, q, $J=6.9$ Hz, H-14), 3.38 (1H, br s, H-9), 3.56 (1H, d, $J=8.7$ Hz, H-1), 4.14 (1H, m, H-2), 4.49 (1H, d, $J=4.9$ Hz, H-11), 5.78 (1H, ddd, $J=6.0$, 4.9, 3.2 Hz, H-12), 5.92 (1H, s, H-6), 6.57 (1H, br s, D_2O exchangeable, OH), 6.91 (1H, d, $J=6.0$ Hz, D_2O exchangeable, OH-12), 6.96 (1H, br s, D_2O exchangeable, OH); ^{13}C NMR (Pyridine-d_5, 75 MHz), δ 16.8 (C-15), 17.4 (C-17), 18.2 (C-16), 22.7 (C-18), 31.7 (C-14), 32.2 (C-4), 40.7 (C-3), 46.5 (C-10), 47.8 (C-8), 48.4 (C-13), 53.0 (C-9), 69.3 (C-12), 70.4 (C-2), 84.8 (C-11), 85.2 (C-1), 120.5 (C-6), 171.0 (C-5), 177.3 (C-19), 199.5 (C-7); FABMS m/z 373 ($[M+Na]^+$, 46), 351 ($[M+H]^+$, 40); HRFABMS m/z 351.1806 (MH^+ calcd for $C_{19}H_{27}O_6$, 351.1808).

4.2.2. Eurycomalide B (2). Colorless syrup; $[\alpha]_D^{25}$ −25.0° (c 0.042, MeOH); UV (MeOH) λ_{max} (log ε) 283 (4.17) nm; IR (KBr) ν_{max} 3424, 2910, 1743, 1658, 1462, 1276 cm^{-1}; ^1H NMR (Pyridine-d_5, 300 MHz), δ 1.05 (1H, d, $J=7.0$ Hz, CH_3-19), 1.77 (3H, s, CH_3-18), 1.79 (3H, s, CH_3-16), 1.89 (3H, s, CH_3-17), 2.60 (1H, d, $J=3.1$ Hz, H-9), 3.33 (1H, m, H-13), 3.41 (1H, s, H-14), 3.94 (1H, d, $J=7.5$ Hz, H-1), 4.50 (1H, m, H-12), 4.70 (1H, d, $J=7.5$ Hz, H-2), 5.99 (1H, m, H-11), 6.08 (1H, s, H-6), 6.21 (1H, s, H-3), 6.88 (1H, d, $J=5.6$ Hz, D_2O exchangeable, OH-11); ^{13}C NMR (Pyridine-d_5, 75 MHz), δ 16.2 (C-17), 16.8 (C-19), 19.8 (C-16), 23.6 (C-18), 32.2 (C-13), 46.0 (C-10), 46.8 (C-8), 47.7 (C-9), 53.3 (C-14), 69.6 (C-11), 71.5 (C-2), 83.1 (C-1), 84.9 (C-12), 121.2 (C-6), 131.0 (C-4), 137.6 (C-3), 163.0 (C-5), 177.3 (C-15), 199.5 (C-7); FABMS m/z 349 ($[M+H]^+$, 11), 313 (21); HRFABMS m/z 349.1650 (MH^+ calcd for $C_{19}H_{25}O_6$, 349.1651).

4.2.3. 13β, 21-Dihydroxyeurycomanol (3). Colorless powder (MeOH); mp 260–262 °C; $[\alpha]_D^{25}$ +51.4° (c 0.12, MeOH); IR (KBr) ν_{max} 3465, 1735, 1648, 1569, 1393, 1263 cm^{-1}; ^1H NMR (Pyridine-d_5, 300 MHz), δ 1.60 (3H, s, CH_3-18), 1.76 (3H, s, CH_3-19), 1.94 (1H, dd, $J=14.7$, 12.8 Hz, H-6), 2.11 (1H, d, $J=14.7$ Hz, H-6), 2.75 (1H, d, $J=12.8$ Hz, H-5), 3.32 (1H, s, H-9), 4.01 (2H, d, $J=8.4$ Hz, H-1 and H-20), 4.60 (1H, d, $J=11.4$ Hz, H-21), 4.60 (2H, m, H-2 and H-12), 5.00 (1H, d, $J=11.4$ Hz, H-21), 5.02 (1H, br s, H-7), 5.19 (1H, d, $J=8.4$ Hz, H-20), 5.43 (1H, s, H-15), 5.77 (1H, br s, H-3), 7.28 (1H, br s, D_2O exchangeable, OH), 7.82 (1H, br s, D_2O exchangeable, OH); ^{13}C NMR (Pyridine-d_5, 75 MHz), δ 11.3 (C-19), 21.1 (C-18), 25.6 (C-6), 41.5 (C-10), 41.9 (C-5), 47.5 (C-9), 53.5 (C-8), 66.8 (C-20), 67.6 (C-21), 70.6 (C-15), 72.7 (C-2), 75.3 (C-7), 78.0 (C-13), 78.2 (C-14), 79.7 (C-12), 83.8 (C-1), 110.1 (C-11), 126.9 (C-3), 135.4 (C-4), 173.5 (C-16); FABMS m/z 467 ($[M+Na]^+$, 5), 413 (10); HRFABMS m/z 467.1526 ($[M+Na]^+$ calcd for $C_{20}H_{28}NaO_{11}$, 467.1529).

4.2.4. 5α, 14β, 15β-Trihydroxyklaineanone (4). Colorless syrup; $[\alpha]_D^{25}$ +31.8° (c 0.041, MeOH); UV (MeOH) λ_{max} (log ε) 227 (3.93) nm; IR (KBr) ν_{max} 3380, 1739, 1655, 1565, 1460 cm^{-1}; ^1H NMR (Pyridine-d_5, 300 MHz), δ 1.75 (3H, s, CH_3-19), 1.87 (1H, d, $J=7.2$ Hz, CH_3-21), 1.98 (3H, s, CH_3-18), 2.11 (3H, s, CH_3-20), 2.49 (2H, s, H-6), 3.13 (1H, q, $J=7.2$ Hz, H-13), 3.94 (1H, d, $J=2.3$ Hz, H-9), 4.45 (1H, br s, H-12), 5.01 (1H, br s, H-7), 5.22 (1H, s, H-1), 5.74 (1H, br s, H-11), 5.99 (1H, d, $J=5.4$ Hz, D_2O exchangeable, OH), 6.18 (1H, s, H-3), 6.18 (1H, s, D_2O exchangeable, OH), 6.37 (1H, d, $J=3.6$ Hz, H-15), 6.78 (1H, d, $J=3.9$ Hz, D_2O exchangeable, OH), 6.88 (1H, d, $J=3.9$ Hz, D_2O exchangeable, OH), 7.24 (1H, br s, D_2O exchangeable, OH); ^{13}C NMR (Pyridine-d_5, 100 MHz), δ 14.5 (C-21), 16.7 (C-19), 18.4 (C-20), 20.2 (C-18), 32.2 (C-6), 37.0 (C-13), 38.1 (C-9), 44.1 (C-8), 50.8 (C-10), 72.0 (C-15), 75.0 (C-11), 75.4 (C-5), 77.6 (C-14), 78.0 (C-12), 79.4 (C-1), 82.3 (C-7), 125.2 (C-3), 165.2 (C-4), 175.3 (C-16), 201.0 (C-2); FABMS m/z 413 ($[M+H]^+$, 17); HRFABMS m/z 413.1808 (MH^+ calcd for $C_{20}H_{29}O_9$, 413.1812).

5. Biological assays

5.1. In vitro cytotoxicity assay

Cytotoxic assays were performed as described in Rubinstein et al.[42] The cell lines used were human lung cancer (A549) and human breast cancer (MCF-7). Cytotoxicity, ED_{50} for each cell line, is the concentration of compound that causes a 50% reduction in adsorbance at 562 nm relative to untreated cells using the sulforhodamine B assay.

5.2. Anti-HIV evaluation

Inhibition of HIV replication was evaluated in the H9 lymphocyte T cell line using a p24 antigen ELISA assay as previously described.[43] P24 antigen is a core protein of HIV and therefore is an indirect measure of virus present in the supernatants. AZT was also assayed during each experiment as a positive drug control.

5.3. Antimalarial assay

In vitro antimalarial drug susceptibility was assayed as previously described[44] using a modified procedure first published by Desjardins et al.[45] with modifications developed by Milhous et al.[46] The assay is based on the incorporation of radiolabeled hypoxanthine by the parasite with known antimalarials mefloquine and choroquine assayed in parallel as positive controls.

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