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ORIGINAL ARTICLE

Quassinoids from the Root of *Eurycoma longifolia* and Their Antiproliferative Activity on Human Cancer Cell Lines

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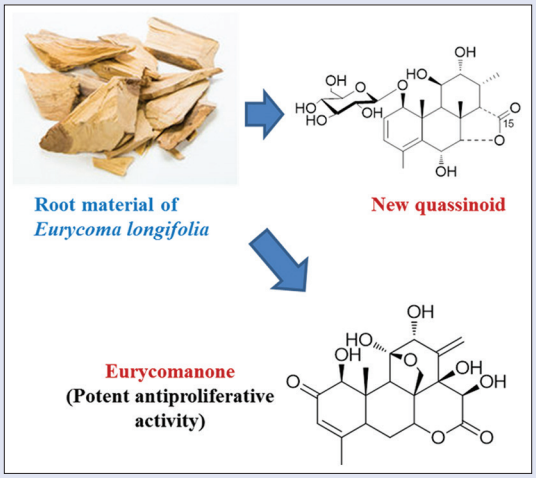
ABSTRACT

Background: The roots of *Eurycoma longifolia* Jack have traditionally been used as an aphrodisiac tonic besides the other remedies for boils, fever, bleeding gums, and wound ulcer. Recently, the antiproliferative activity of *E. longifolia* has been reported and remained attractive to natural chemists. **Objective:** The objective of this study was to study on antiproliferative compounds from the root of *E. longifolia*. **Materials and Methods:** Column chromatography was used to separate individual compounds, spectroscopic data including nuclear magnetic resonances and mass spectrometry were analyzed to determine the chemical structure of the isolates and for biological testing, antiproliferative activity of compounds was tested on seven human cancer cell lines (KATO III, HCT-15, Colo205, HepG2, PC-3, Jurkat, HL-60) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. **Results:** Nine quassinoids including a new C_{19} longilactone-type quassinoid glycoside were characterized from the roots of the title plant. Among them, the major quassinoid eurycomanone exhibited selectively potential antiproliferative activities on the leukemia cell lines (HL-60 and Jurkat) and had very low toxic effects on normal skin fibroblast cell line (NB1RGB). **Conclusion:** The current study reveals one new quassinoid glycoside and the potential active component (eurycomanone) from *E. longifolia* for the leukemia treatment.

Key words: Antiproliferative activity, *Eurycoma longifolia*, leukemia cells, quassinoid, *Simaroubaceae*

SUMMARY

- Nine quassinoids (1-9) including one new quassinoid glycoside (9) and eight known ones were isolated from the roots of *Eurycoma longifolia*
- The structure of the new quassinoid 9 was determined by extensive chemical and spectroscopic analyses
- The major quassinoid, eurycomanone (3), exhibited selectively potential antiproliferative activities on both Jurkat and HL-60 leukemia cells and had very low toxic effects on normal skin fibroblast cell line (NB1RGB).



Abbreviations used: COSY: Correlation spectroscopy; HMBC: Heteronuclear multiple-bond correlation; HMQC: Heteronuclear multiple quantum correlation; NMR: Nuclear magnetic resonance; NOESY: Nuclear Overhauser enhancement spectroscopy; TLC: Thin layer chromatography.

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INTRODUCTION

Eurycoma longifolia Jack (*Simaroubaceae*) is a flowering shrub plant native to Indonesia (commonly known as Pasak Bumi), Malaysia (called as Tongkat Ali), and to a lesser extent, Thailand, Vietnam (with local name "Cay Ba Binh"), and Laos. Its roots have traditionally been used as an aphrodisiac tonic besides the other remedies for boils, fever, bleeding gums, and wound ulcer.[1] Previous phytochemical studies on the root of *E. longifolia* have led to the isolation of nearly seventy quassinoids related to triterpene, considered as principal active constituents and responsible for typical bitter taste, together with several phenolic,[2,3] typical triterpenes such as melianone,[4] squalene-type triterpene,[5] and canthin-6-one alkaloid like picrasidine L,[6] some of which have potential pharmacological and therapeutic properties.[2,3]

In our ongoing research regarding anticancer phytotherapy, we previously reported the potent antiproliferative activity of alkannin derivatives from *Alkanna tinctoria*,[7,8] crocin from saffron (*Crocus sativus*),[9,10] triterpenes from *Eriobotrya japonica*,[11] and coumarins from *Mammea siamensis*.[12] As part of the ongoing study, our current phytochemical investigation on antiproliferative constituents from the root of the title plant resulted in the isolation of nine quassinoids including a novel one. This paper herein deals with the structural elucidation of the new quassinoid and the antiproliferative activity of isolated quassinoids using seven human cancer cells.

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MATERIALS AND METHODS

General procedures

Optical rotations were obtained using a P-1020 digital polarimeter (JASCO, Easton, USA). NMR spectra were recorded on a JEOL ECX 400 NMR spectrometer (JEOL, Tokyo, Japan). High-resolution electrospray ionization (HR-ESI) time-of-flight mass spectrometry (MS) experiments utilized a JEOL AccuTOF™ LC 1100 mass spectrometer (JEOL, Tokyo, Japan). High-performance liquid chromatography (HPLC) analysis of sugar was run on an Agilent 1100 Series HPLC system (Agilent, Santa Clara, CA, USA) equipped with a YMC-Pack NH_2 column (250 mm × 4.6 mm i.d., NH12S05-2546WT, YMC Co. Ltd., Kyoto, Japan) and an optical rotation detector JASCO OR-2090 (JASCO, Easton, USA). Column chromatography was performed on silica gel 60 (230–400 mesh, Nacalai Tesque Inc., Kyoto, Japan) and YMC ODS-A gel (50 µm, YMC Co. Ltd., Kyoto, Japan). Thin-layer chromatography (TLC) was performed on Kieselgel 60 F_{254} and TLC silica gel 60 RP-18 F_{254S} (Merck, Darmstadt, Germany) plates. Spots were visualized by spraying with 1% $Ce(SO_4)_2$-10% aqueous H_2SO_4 solution, followed by heating.

Plant material

The roots of *E. longifolia* (powder form) were purchased from Malaysian medicinal market in 2012 and authenticated by one of the authors (YS). A voucher specimen (EL-2012) has been deposited in the Department of Pharmacognosy, Nagasaki International University.

Extraction and isolation

The root powder (500 g) of *E. longifolia* was extracted with 95% EtOH (2.0 L × 4 times) at 40°C under sonication. The combined extracts were concentrated to give yellow syrup (14.2 g), which was then suspended in water (250 ml) following partitioning with EtOAc (250 ml × 3 times) and water-saturated n-BuOH (250 ml × 3 times) to give EtOAc soluble extract (4.1 g) and n-BuOH soluble extract (4.5 g). The EtOAc extract (4.1 g) was subjected to a silica gel column eluted with $CHCl_3$-MeOH (15:1, v/v) to give nine fractions (fr.1.1–fr.1.9). Fr.1.7 (610 mg) was further chromatographed over a reversed-phase (RP) column eluted with MeOH-H_2O (1:2, v/v) to yield 1 (12 mg) and 2 (23 mg). Likewise, fr.1.9 (1050 mg) was applied to a RP column eluted with MeOH-H_2O (1:2, v/v) to obtain 3 (28 mg) and 4 (36 mg). BuOH extract (4.5 g) was subjected to a RP column eluted with MeOH-H_2O (2:5, v/v) to afford four fractions (fr.2.1–fr.2.4). Then, fr.2.2 (300 mg) was further chromatographed over a silica gel column eluted with $CHCl_3$-MeOH (6:1, v/v) to yield 6 (14 mg), 7 (9 mg), 9 (13 mg), and additional 3 (65 mg). Fr.2.3 (600 mg) was loaded onto a RP column eluted with MeOH-H_2O (2:5, v/v) to give 5 (18 mg) and a subfraction (170 mg), which was subsequently applied to a silica gel column eluted with $CHCl_3$-MeOH (5:1, v/v) to yield 8 (9 mg) and additional 4 (58 mg).

Compound 8 ($\Delta^{4,5}$-14-hydroxyglaucarubol): colorless solid; +68° (*C* 0.45, MeOH); HR-ESI-MS m/z: 413.1819 $[M + H]^+$ (calcd for $C_{20}H_{29}O_9$, 413.1812); ^{1}H-NMR (CD_3OD, 400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) data [Table 1].

Table 1: ^{1}H- and ^{13}C-NMR data for 8 and 9 in CD_3OD

Position	8		9	
	δ_C	δ_H	δ_C	δ_H
1	84.2	3.28 (^{1}H, d, J=5.2 Hz)	82.0	4.42 (^{1}H, br s)
2	68.2	3.63 (1H, m)	126.8	5.63 (^{1}H, d, J=10.4 Hz)
3	41.4	2.01 (^{1}H, dd, J=11.2, 4.0 Hz) 2.23 (^{1}H, dd, J=11.2, 2.4 Hz)	133.4	6.17 (^{1}H, d, J=10.4 Hz)
4	128.3		142.3	
5	129.6		50.4	2.24 (^{1}H, d, J=10.8 Hz)
6	28.1	2.40 (^{1}H, dd, J=15.6, 7.2 Hz) 2.80 (^{1}H, dd, J=15.6, 4.0 Hz)	65.4	4.06 (^{1}H, dd, J=10.8, 3.2 Hz)
7	77.3	4.61 (^{1}H, dd, J=7.2, 4.0 Hz)	88.1	3.96 (^{1}H, d, J=3.2 Hz)
8	53.9		44.3	
9	50.7	2.25 (1H, s)	42.3	1.78 (^{1}H, br s)
10	45.0		44.2	
11	110.5		71.7	5.12 (^{1}H, br s)
12	79.6	3.30 (^{1}H, d, J=4.0 Hz)	76.5	3.53 (^{1}H, br s)
13	42.0	2.10 (^{1}H, dt, J=7.6, 4.0 Hz)	28.0	2.24 (^{1}H, m)
14	76.5		56.9	2.20 (^{1}H, d, J=5.2 Hz)
15	71.5	4.45 (^{1}H, s)	179.4	
16	175.8			
18	19.8	1.61 (^{3}H, s)	114.7	5.03 (^{1}H, br s) 5.31 (^{1}H, br s)
19	18.4	1.35 (3H, s)	13.0	1.01 (^{3}H, s)
20	67.9	3.97 (^{1}H, d, J=10.0 Hz) 3.86 (^{1}H, d, J=10.0 Hz)	20.9	1.46 (^{3}H, s)
21	13.1	1.13 (^{3}H, d, J=7.6 Hz)	14.7	1.27 (^{3}H, d, 8.0 Hz)
1′			100.7	4.47 (^{1}H, d, J=8.0 Hz)
2′			75.1	3.25
3′			78.0	3.30
4′			71.6	3.25-3.30
5′			77.8	3.51
6′			62.6	3.54 (^{1}H, dd, J=12.0, 4.8 Hz) 3.75 (^{1}H, br d, J=12.0)

Assignments were confirmed by COSY, HMQC, HMBC, and NOESY spectra

Compound 9: colorless solid; +26° (*C* 0.30, MeOH); HR-ESI-MS m/z: 513.2348 $[M + H]^+$ (calcd for $C_{25}H_{37}O_{11}$, 513.2336); ^{1}H-NMR (CD_3OD,400 MHz) and ^{13}C-NMR (CD_3OD, 100 MHz) data [Table 1].

Acid hydrolysis of 9

A solution of 9 (4.0 mg) in 1.0 M HCl (5.0 ml) was heated under reflux for 4 h. After cooling, the reaction mixture was poured into ice water and neutralized with Amberlite IRA-400 (hydroxyl form), and the resin was removed by filtration. Then, the filtrate was concentrated *in vacuo* to dryness, followed by partition between EtOAc and H_2O. The aqueous layer after filtration was subjected to HPLC analysis with the mobile phase of CH_3CN-H_2O (80:20, v/v) at flow rate 0.40 ml/min. D-glucose was detected at 9.6 min with positive optical rotation and compared with the authentic sample.

Cell culture and sample treatment

All cell lines were obtained from RIKEN BioResource Center Cell Bank. HepG2 and NB1RGB were maintained in Dulbecco's modified Eagle's medium and minimum essential medium-α, respectively. The other cell lines were maintained in RPMI1640 medium. All these media were supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin and were then incubated at 37°C under 5% CO_2 in fully humidified conditions. For the cell treatment, dimethyl sulfoxide (DMSO) concentrations in the cell culture medium did not exceed 0.2% (v/v) and the controls were always treated with the same amount of DMSO as used in the corresponding experiments.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[11,12] In brief, the cells were seeded in 96-well plates at a density of 1×10^4 cells/well for suspension cells or 0.5×10^4 cells/well for attached cells. After incubation for 24 h, the cells were treated with each compound at various concentrations for 48 h. At the end of treatment, MTT solution was added to each well, and the cells were incubated for another 4 h. The precipitated MTT-formazan was dissolved with 0.04 N HCl-isopropanol, and the amount of formazan was measured at 595 nm using a microplate reader (iMark, BioRad, Tokyo, Japan). Cell viability was expressed as a percentage of the control culture.

RESULTS AND DISCUSSION

The ethanolic extract of the root of *E. longifolia* was suspended in water and then partitioned with EtOAc and n-BuOH, followed by various column chromatography to afford 13,21-dihydroeurycomanone (1),[13] 14.15 β-dihydroxyklaineanone (2),[2] eurycomanone (3),[2] eurycomanol (4),[14] 2-hydroxylongilactone-4(18)-ene (5),[15] longilactone (6),[2] eurycomaoside (7),[16] $\Delta^{4,5}$-14-hydroxyglaucarubol (8),[17] and especially one new quassinoid (9) [Figure 1].

Compound 8 was obtained as colorless solid and has the molecular formula $C_{20}H_{28}O_9$ based on HR-MS measurement. The ^{1}H and ^{13}C NMR spectroscopic data of 8 [Table 1] suggested a C_{20} quassinoid structure similar to 13,21-dihydroeurycomanol with a lactone carbonyl signal (δ_C 175.8), a double bonds (δ_C 129.6, 128.3), and seven oxygenated carbon groups (δ_C 84.2, 79.6, 77.3, 76.5, 71.5, 68.2, 67.9).[2,13] Further analyses and comparison of NMR data of 8 with those in literature led us to confirm the structure of 8 as $\Delta^{4,5}$-14-hydroxyglaucarubol,[17] which was recently reported when we were doing this research.

Compound 9, a colorless solid, has the molecular formula $C_{20}H_{28}O_9$ as deduced by HR-MS experiment. The NMR data of 9 suggested a quassinoid glycoside. In detail, the ^{1}H NMR (CD_3OD) spectrum of 9 [Table 1] showed four olefinic protons (δ 6.17, 5.63, 5.31, 5.03), five oxymethine protons (δ 5.12, 4.42, 4.06, 3.96, 3.53), a secondary methyl (δ 1.27), and two tertiary methyls (δ 1.46, 1.01), which were attributable to the quassinoid aglycone, and an anomeric proton (δ 4.47) with large coupling constant (J = 8.0 Hz) typical of the β-linked sugar unit. The ^{13}C NMR spectrum of two revealed 25 signals including a set of six signals (δ 100.7, 75.1, 78.0, 71.6, 77.8, 62.6) contributing to a β-glucopyranosyl unit and 19 remaining ones of a C_{19}-quassinoid skeleton, which consisted of a lactone carbonyl (δ 179.4), two double bonds (δ 142.3, 133.4, 126.8, 114.7), five oxymethine carbons (δ 88.1, 82.0, 76.5, 71.7, 65.4), four methines (δ 56.9, 50.4, 42.3, 28.0), two quaternary carbons (δ 44.3, 44.2), a secondary methyl (δ 14.7), and two tertiary methyls (δ 20.9, 13.0), respectively. Further inspection of the ^{1}H and ^{13}C NMR data of 9 found that they were similar to those of eurycomaoside except for the signals belonging to ring A, which were consistent with $\Delta^{1,4(18)}$-diene frame. The acid hydrolysis of 9 liberated D-glucose as confirmed

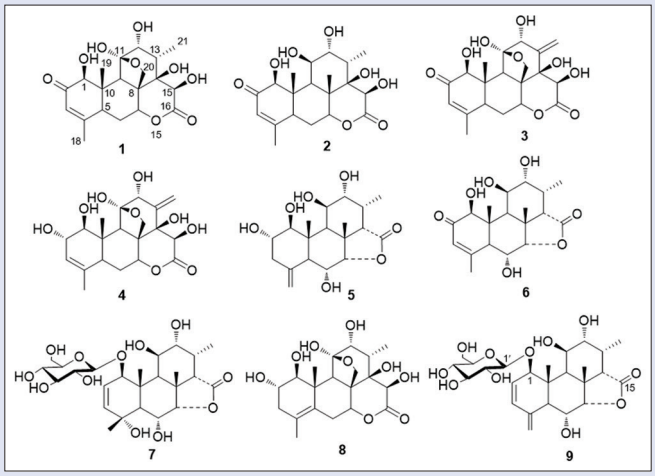

Figure 1: Structures of quassinoids 1–9

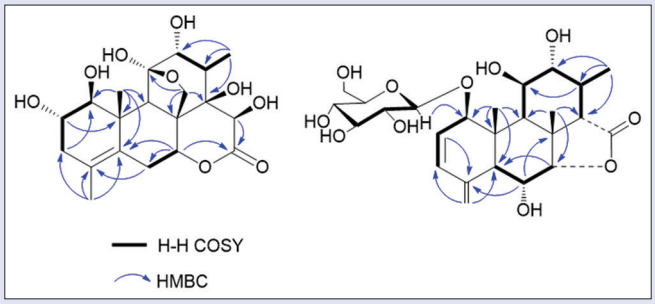

Figure 2: Key HMBC and COSY correlations of 8 and 9

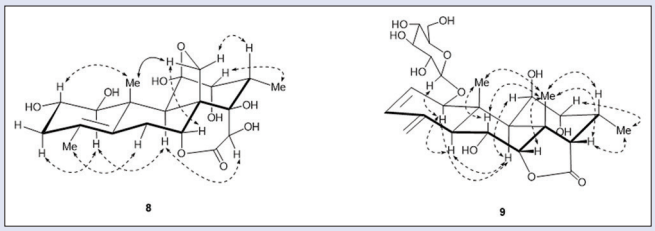

Figure 3: Relative configuration of 8 and 9 with key nuclear Overhauser effect correlations

Table 2: Cell viability of 1-9 measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay using human cancer and normal cell lines

	Cell viability (%)								
Cell line	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
KATO III	84.5±7.4	75.1±5.4	72.5±9.1	83.2±7.1	88.8±2.7	84.7±2.3	84.0±1.5	88.3±7.6	83.8±4.0
HCT-15	82.6±2.3	36.7±2.4	32.5±0.6	83.8±3.5	92.2±2.7	73.1±0.7	92.0±4.8	90.8±4.1	91.9±4.4
Colo205	70.3±4.4	35.0±1.0	60.8±3.8	82.3±7.4	97.4±5.3	87.6±3.8	89.9±3.8	88.8±3.1	86.5±1.2
HepG2	87.8±3.3	65.7±3.2	76.5±3.4	89.5±0.9	98.8±1.8	92.1±4.1	94.2±4.1	85.9±2.0	85.6±2.5
PC-3	71.7±2.6	42.0±2.7	46.1±3.5	70.3±5.5	84.2±4.7	76.8±3.0	91.2±1.6	89.8±3.4	83.1±3.5
HL-60	56.8±3.5	4.9±0.2	4.0±0.5	61.9±3.5	92.7±7.8	58.6±6.2	90.7±9.1	94.6±3.7	90.3±3.7
Jurkat	75.4±2.3	9.2±0.2	19.8±0.9	87.7±3.4	97.8±5.3	72.1±4.4	98.4±2.4	98.4±1.8	89.7±5.3
NB1RGB	93.7±7.8	84.4±8.5	88.2±9.9	98.0±8.1	105.1±2.6	106.3±5.7	90.6±4.5	87.1±6.4	87.4±8.6

by HPLC analysis. Moreover, the proposed structure of 9 was assured by the HMQC, COSY, and HMBC spectra [Figure 2]. Likewise, the relative stereochemistry of 9 was assigned on the basis of the nuclear Overhauser effect (NOE) spectroscopy spectrum. The transconformation of A/B and B/C junctions was configured due to NOE correlations of H-5/H-1, H-5/H-9, Me-19/H-6, and Me-19/Me-20. In addition, the NOE interactions of H-7/Me-20, H-9/H-11, H-13/Me-20, H-14/Me-20, and H-12/Me-21 further certified the similar stereochemistry between 9 and eurycomaoside (7)[16] as well as longilactone (6)[2] [Figure 3]. Finally, the NOE cross peak of H-1/H-1′ indicated the sugar linkage at C-1 of the quassinoid aglycone. Consequently, the structure of compound 9, one of two quassinoid glycosides including eurycomaoside (7) in nearly 70 quassinoids, was elucidated as depicted in Figure 1.

In this study, we investigated whether the isolated quassinoids had antiproliferative activities against various cancer cell lines. Accordingly, we examined the effect of quassinoids 1–9 on proliferation of seven human cancer cell lines, namely, KATO III (stomach cancer), HCT-15 (colon cancer), Colo205 (colon cancer), HepG2 (hepatoma), PC-3 (prostate cancer), HL-60 (promyelocytic leukemia), and Jurkat (acute T cell leukemia), and the human normal skin fibroblast cell line NB1RGB. Cells were treated with compounds 1–9 at concentrations of 25 μM for 48 h, and cell viability was determined by MTT assay. As shown in Table 2, among nine tested compounds, quassinoids 2 and 3 were found relatively to have strongest antiproliferative activities against all assayed cancer cell lines. The rest of the compounds including the two new constituents were considered to be inactive against the seven cancer cell lines. Noticeably, 14,15 β-dihydroxyklaineanone (2) and the major quassinoid eurycomanone (3) exhibited potent inhibitory effects on the growth of both leukemia cell lines; when treated with 25 μM, compound 2 inhibited cell growth by 95.1% (HL-60) and 90.8% (Jurkat) while compound 3 inhibited cell growth by 96.0% (HL-60) and 80.2% (Jurkat), respectively. Moreover, both compounds had negligible toxic effect on the viability of the normal skin fibroblast cell line NB1RGB. Taken together, these results suggest that 14,15 β-dihydroxyklaineanone (2) and the major quassinoid eurycomanone (3) are potent antiproliferative compounds against leukemia cells. The results could not be readily explained in terms of the structures of quassinoid derivatives.

CONCLUSION

We have identified nine quassinoids including a new C_{19} longilactone-type quassinoid glycoside (9) and antiproliferative testing revealed 14,15 β-dihydroxyklaineanone (2) and the major quassinoid eurycomanone (3) as potent preferential antileukemia agents which may be of potential therapeutic use for the leukemia treatment in the future. Further studies on their mechanism of action and *in vivo* antitumor need to be explored.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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