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Quassinoids from Malaysian *Eurycoma longifolia* significantly increased the expression of the melatonin biosynthesis-related enzyme gene (AANAT)[†]

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Eurycoma longifolia Jack, also known as Tongkat Ali, is a native plant in the family of Simaroubaceae that grows in Southeast Asian rain forests. *E. longifolia* has garnered significant attention due to its profound pharmacological properties (antimalarial, anti-pyretic, antiulcer, cytotoxic and aphrodisiac properties). However, the recent report that the intake of Tongkat Ali supplements improve sleep quality in healthy adults has aroused our interest. Three the most characteristic C₂₀-quassinoids (1–3) were isolated from the roots of *Eurycoma longifolia*, along with eight known secondary metabolites with other structural types (4–11). Their structures were identified by comprehensive analyses of NMR spectroscopy, and HRMS data. The sleep-promoting activity of three quassinoids based on the melatonin (sleep hormone) biosynthesis-related enzyme gene (AANAT) expression test was also evaluated, and the results revealed that quassinoids (1–3) significantly increased AANAT gene expression compared to the control. These results provide new ideas for the development and utilization of the plant for clinical applications of sleep disorders in the future.

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1. Introduction

Eurycoma longifolia Jack (commonly called Tongkat Ali) is a flowering plant in the family Simaroubaceae. It is native to Indochina (Cambodia, Laos, Malaysia, Myanmar, Thailand and Vietnam) and Indonesia (the islands of Borneo and Sumatra).¹ The root of *E. longifolia* has been used in folk medicine in the Southeast Asian region to treat malaria, dysentery, glandular swelling, persistent fever, aches, and sexual insufficiency.² In modern times it has found common use as supplements, as well as food and drink additives.

Insufficient sleep is a pervasive and prominent problem in the modern 24 h society. Medical experts have said that about one-third of the people in the world suffer from sleep disorders. Insufficient sleep is prevalent across various age groups, considered to be a public health epidemic. After decades of

research, the case can be confidently made that sleep loss and sleep disorders have profound and widespread effects on human health.³ It leads to increased incidences of cardiovascular morbidity, increased chances of diabetes mellitus, obesity, derailment of cognitive functions, vehicular accidents, and increased accidents at workplaces.²

The latest research indicated the intake of Tongkat Ali supplement containing about 402 mg of Tongkat Ali powder a day enhanced mood state and consequently improve the sleep quality in a healthy population.⁴ These unique properties make Tongkat Ali (*E. longifolia* Jack) plant valuable for potential applications, and it is worth further exploring the active substances in *E. longifolia*. Evaluation of its sleep-promoting activity of these compounds can provide valuable insights into the role of *E. longifolia* in improving the sleep disorders that is the worldwide public health epidemic.

In this study, we investigated further related constituents from *n*-hexane-, dichloromethane- and ethyl acetate-soluble extract of the water extract of the roots of *E. longifolia*, and isolated three quassinoids (1–3), along with several known secondary metabolites (4–11) are displayed in Fig. 1. We herein describe the isolation, structure elucidation of these compounds, and evaluation of the sleep-promoting activity of the most characteristic chemical components (quassinoids) of this plant based on the gene expression test of enzymes related to melatonin (sleep hormone) biosynthesis.

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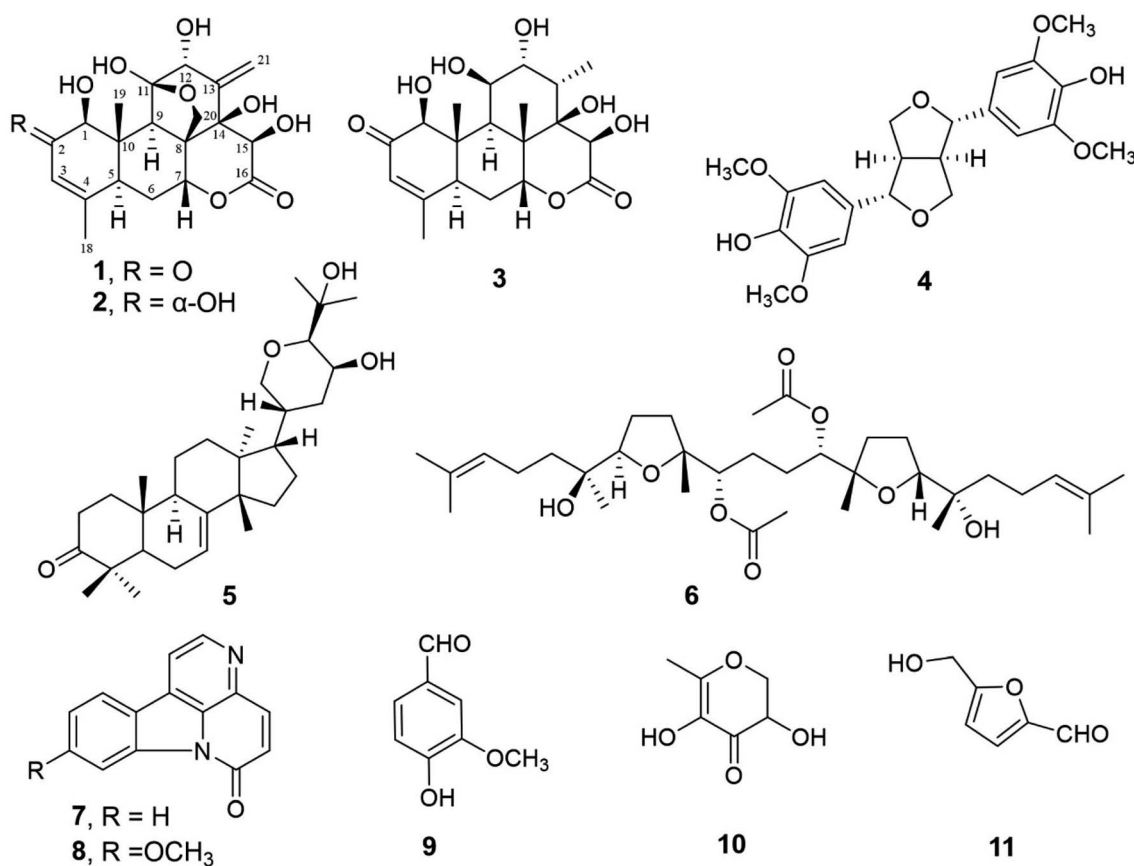


Fig. 1 The chemical structures of secondary metabolites from *E. longifolia* (1–11).

2. Results and discussion

2.1. Chemistry

The HPLC chromatograms of three quassinoids (1–3) were presented in Fig. 2a. Three peaks were observed at 11.57 (purple), 12.89 (red), and 16.29 min (black), which

corresponded to eurycomanone (1) and eurycomanol (2) and 14,15 β -dihydroxyklaineanone (3), respectively. High-Resolution Mass Spectrum (HRMS) shows the observed $[M + H]^+$ peak at m/z 409.1514 (calcd for C₂₀H₂₅O₉, 409.1499) of 1, 411.1647 (calcd for C₂₀H₂₇O₉, 411.1655) of 2 and 397.1862 (calcd for C₂₀H₂₉O₈, 397.1862) of 3 in Fig. 2b.

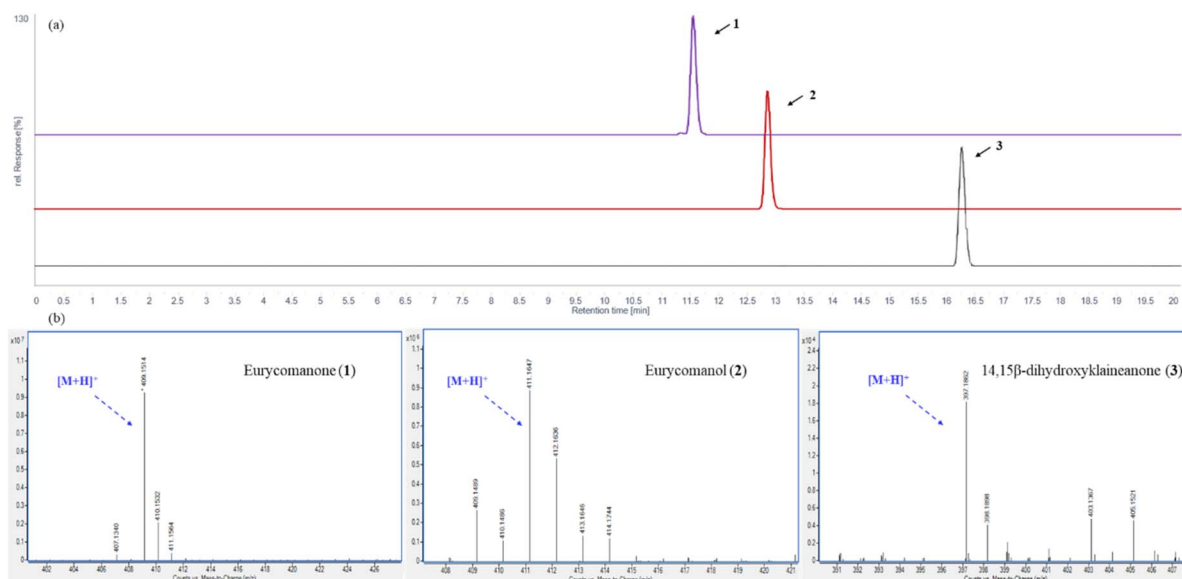


Fig. 2 (a) HPLC analysis result of three quassinoids (1–3); (b) HRMS spectra of the three quassinoids (1–3).



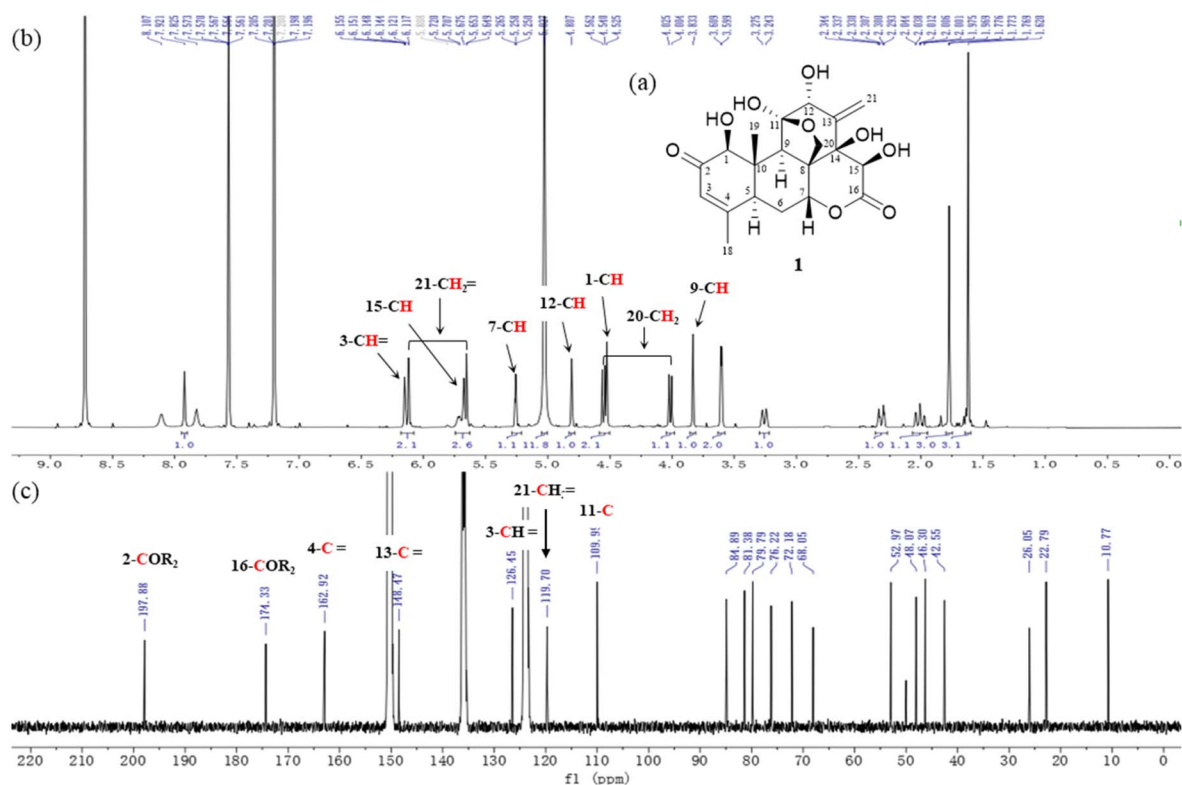


Fig. 3 (a) The chemical structure, (b and c) ^1H and ^{13}C NMR spectra of eurycomanone (1).

Compound (1) was obtained as colorless needle with the molecular formula $\text{C}_{20}\text{H}_{25}\text{O}_9$, which was determined by the ion peaks at m/z 409.1514 $[\text{M} + \text{H}]^+$ observed by HR-ESIMS. In the ^1H and ^{13}C NMR spectra of 1 revealed the presence of two methyl groups (δ_{H} 1.62 and 1.77, δ_{C} 10.8 and 22.8), two methine (δ_{H} 3.26, δ_{C} 42.6) and (δ_{H} 3.83, δ_{C} 48.1), a methylene (δ_{H} 2.21 and

Table 1 ^1H and ^{13}C NMR spectroscopic data for 1–3 (δ in ppm, J in Hz)^a

No.	1^b δ_{C} , type δ_{H}		2^b δ_{C} , type δ_{H}		3^c δ_{C} , type δ_{H}	
1	84.9 CH	4.53, s	84.1, CH	4.07, d (8.2)	82.6, CH	4.04, s
2	197.9, C	—	73.1, CH	4.66, m	198.2, C	—
3	126.4, CH	6.15, br s	127.4, CH	5.82, br s	124.3, CH	6.09, br s
4	162.9, C	—	135.4, C	—	164.5, C	—
5	42.6, CH	3.26, br d (12.8)	42.0, CH	2.85, br d (12.4)	43.6, CH	2.87, br d (12.8)
6	26.1, CH ₂	2.21, ddd (14.8, 12.8, 2.8)	26.0, CH ₂	1.93, br t (14.4)	26.1, CH ₂	2.10, m
7	72.2, CH	5.26, t (2.8)	72.3, CH	5.19, br s	81.5, CH	4.63, t (3.2)
8	53.0, C	—	53.1, C	—	48.2, C	—
9	48.1, CH	3.83, s	48.3, CH	3.58, s	45.0, CH	2.11, d (3.2)
10	46.3, C	—	42.7, C	—	43.0, C	—
11	109.9, C	—	110.2, C	—	73.6, CH	4.88, br s
12	81.4, CH	4.81, s	81.5, CH	4.83, s	77.0, CH	3.91, t (2.8)
13	148.5, C	—	149.0, C	—	35.8, CH	2.43, dq (2.8, 7.3)
14	79.8, C	—	79.8, C	—	75.8, C	—
15	76.2, CH	5.67, s	76.9, CH	5.55, s	71.3, CH	5.33, s
16	174.3, C	—	174.3, C	—	175.9, C	—
18	22.8, CH ₃	1.77, s (1H)	21.7, CH ₃	1.64, br s	22.8, CH ₃	1.97, s
19	10.8, CH ₃	1.62, s (1H)	11.3, CH ₃	1.76, s	12.4, CH ₃	1.18, s
20	68.1, CH ₂	4.01, d (8.8)	68.3, CH ₂	4.07, d (8.2)	17.4, CH ₃	1.51, s
21	119.7, CH ₂	5.65, d (1.6)	119.7, CH ₂	5.66, d (1.6)	12.5, CH ₃	1.22, d (7.2)
		6.12, d (1.6)		6.13, d (1.6)		

^a Overlapped signals were reported without designating multiplicity. ^b Measured in $\text{C}_5\text{D}_5\text{N}$, 400 MHz for ^1H NMR, 100 MHz for ^{13}C NMR. ^c Measured in CDCl_3 , 400 MHz for ^1H NMR, 100 MHz for ^{13}C NMR.



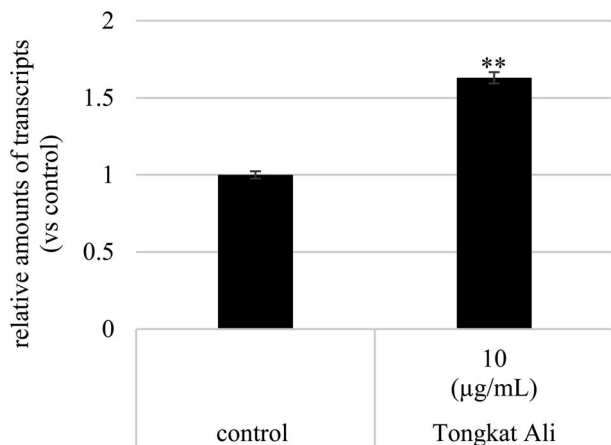


Fig. 5 AANAT gene expression activity of the extract of *E. longifolia*.

the effect of making it go to sleep. Melatonin has the effect of inducing natural sleep by switching between wakefulness and sleep. In addition to inducing sleep, melatonin has antioxidant effects to promote cellular metabolism, and is thought to have various effects on preventing disease and aging. Melatonin biosynthesis in the pineal gland starts from tryptophan and involves four sequential enzymatic steps to render 5-hydroxytryptophan, 5-hydroxytryptamine (serotonin), *N*-acetylserotonin. The certain amount of serotonin goes through acetylation by the first enzyme in melatonin synthesis, arylalkylamine *N*-acetyltransferase (AANAT), to get *N*-acetylserotonin (NAS), which in turn is converted to melatonin with the help of the last enzyme involved in this process, hydroxyindole *O*-methyltransferase (HIOMT), which catalyses the *O*-methylation of NAS by *S*-adenosyl methionine to form melatonin.²⁶

In this study, we firstly investigated the influence of *E. longifolia* extract on melatonin biosynthesis by AANAT, which is a rate-limiting enzyme in melatonin biosynthesis. The expression

of AANAT gene was significantly 1.6 times higher than control when *E. longifolia* extract applied to SH-SY5Y cells. This result indicate that the extract of *E. longifolia* have a possible to increase melatonin biosynthesis by up-regulating AANAT genes (Fig. 5).

2.2.2. AANAT expression inducing activity of quassinoids.

To evaluate whether the most characteristic chemical components, quassinoids (1–3) from *E. longifolia* enhance melatonin biosynthesis, the effect of AANAT gene expression was investigated. As a result, it was observed that eurycomanone (1) and eurycomanol (2) and 14,15β-dihydroxyklaineaneone (3) significantly increased AANAT gene expression compared to the control (Fig. 6). The findings strongly indicate that the upregulation of AANAT gene expression can be attributed to the presence of eurycomanone, eurycomanol and 14,15β-dihydroxyklaineaneone components in *E. longifolia* extract.

The quassinoids are a fascinating class of highly oxygenated degraded triterpene natural product, and many quassinoids have shown the exciting biological properties, including anti-malarial, anti-inflammatory, antiviral, neuroprotective and antifeeding-particularly potent anti-cancer activity.^{27,28} In a controlled trial on the effect of Tongkat Ali supplement intake on the sleep quality in Japanese healthy adults, statistical analysis showed that a significant improvement sleep quality was observed only in the Tongkat Ali group (intaking 402 mg of Tongkat Ali power a day) over a 4 week period, compared with the placebo group.⁴ This finding aligns with the present study, which, for the first time, demonstrates that three C₂₀-type quassinoids isolated from *E. longifolia* significantly upregulate the expression of the AANAT gene, a rate-limiting enzyme critical for the biosynthesis of melatonin (the sleep hormone), thereby revealing the potential of quassinoids in improving and treating “sleep disorders”. However, based on the number of carbon atoms involving the construction of their basic scaffolds, quassinoids are commonly categorized into six distinct groups: C₂₆, C₂₅, C₂₂, C₂₀, C₁₉, and C₁₈ types.^{5,27} The C₂₀-type

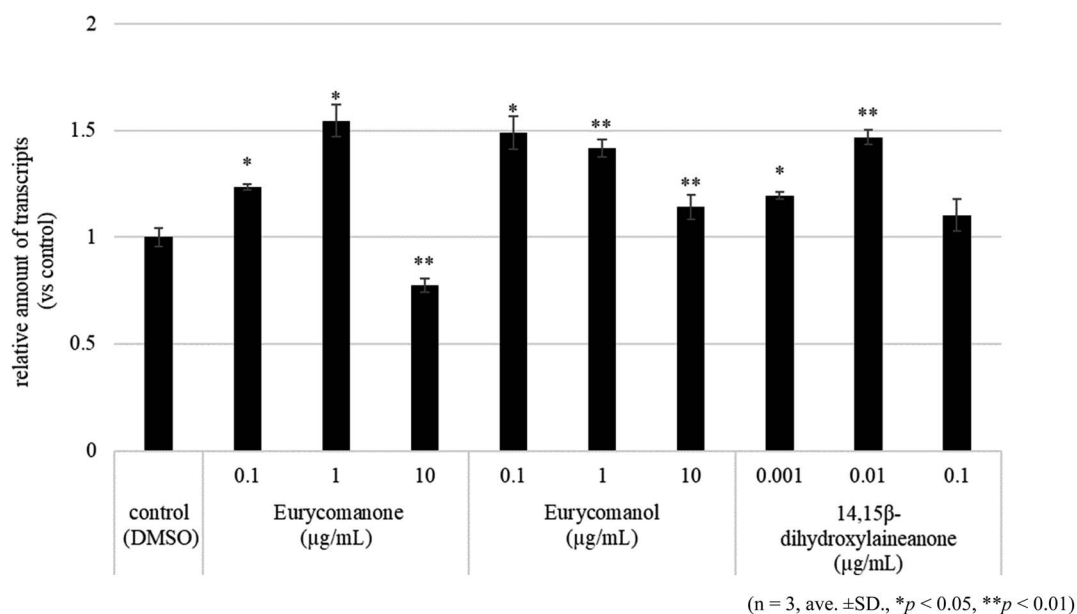


Fig. 6 Enzyme gene (AANAT) expression activity of quassinoids (1–3).



skeleton structure isolated in this study, which is characterized by a tetracyclic ring system often containing a d-ring lactone. Investigating further the structural–activity correlation for six types of quassinoids involved in upregulating AANAT gene activity will serve as a significant driving force for our ongoing exploration of additional quassinoid types from this plant.

Quassinoids are the main components responsible for various biological activities in *E. longifolia* extract, and we speculate that they have hypnotic effects. We observed that *E. longifolia* extract and its isolated product significantly increased the expression of AANAT gene compared with the control in SH-SY5Y cells line. Although the preliminary findings are promising, further extensive studies are required, such as using the three-dimensional structure and molecular docking of the target enzyme combined with computer-aided design to identify their binding sites (associated amino acid residues) of quassinoids to target receptor. Additionally, based on increased AANAT gene expression, investigating elevated levels of its acetylation product, *N*-acetyl-5-hydroxytryptamine (NAS) and ultimately the increased intracellular melatonin content, are necessary to elucidate the mechanism by which it improve the sleep quality in healthy adults.⁴ These unique activities make *E. longifolia* a potentially valuable insight for developing natural products or sleep supplements that enhance sleep.

3. Conclusions

In conclusion, three most characteristic C₂₀-type quassinoids (1–3) were successfully isolated from *E. longifolia*, and their chemical structures and stereochemistry were elucidated by spectroscopic methods. The effects of three components from *E. longifolia* on the melatonin biosynthesis-related enzyme AANAT were investigated. Eurycomanone, eurycomanol and 14,15β-dihydroxykaineanone significantly increased the expression of the rate-limiting enzyme AANAT gene for melatonin synthesis on SH-SY5Y cell compared to the control. These results strongly indicated that the C₂₀-type quassinoids (1–3) contained in *E. longifolia* may be expected to promote melatonin biosynthesis by up-regulating the AANAT gene. This study helps to reveal the second metabolites from *E. longifolia* can yield promising drug candidates for improving and treating “sleep disorders” and to deepen our understanding of the existence of a class of highly oxygenated degraded triterpenoids in *E. longifolia*. The use of C₂₀-type quassinoids from *E. longifolia* which have potential sleep-promoting activities will have broad application prospects in functional food and medicine. Future studies will focus on exploring quassinoids with stronger gene up-regulation ability, investigating changes in melatonin content both inside and outside the cell based on its activity, as well as exploring mechanism of action.

4. Experimental

4.1. General

Organic solvents were purchased from Wako Pure Chemical Industries (Osaka, Japan). Silica gel (75–120 mesh) and RP-C18 silica (38–63 μm) were purchased from Wako Pure Chemical

Industries (Osaka, Japan). Buchi Reveleris® Prep system equipped with NP-silica gel, RP-C₁₈ flash column or Develosil ODS-UG-5 column (5 μm, Ø 20 × 250 mm, Nomura Chemical, Japan), were used for fractionation and fine purification. Optical rotations were recorded on a P-2200 polarimeter (JASCO, Japan). High-performance liquid chromatography (HPLC) was performed using a high-pressure Agilent 1260 Infinity II LC system equipped with UV and ELSD detector (Agilent Technologies Japan, Ltd). All NMR measurements spectra were obtained on a JNM-ECS 400 (400 MHz for ¹H) (JEOL) or DRX-600 spectrometer (600 MHz for ¹H) (Bruker Daltonics, USA). The chemical shifts (ppm) were referenced to the solvent peak of chloroform-*d*₁ (δ_H 7.26/δ_C 77.0), methanol-*d*₄ (δ_H 3.31/δ_C 49.0) or pyridine-*d*₅ (δ_H 7.22/δ_C 123.9) as an internal standard. High-resolution electrospray ionization time-of-flight mass spectra (HRESITOFMS) were recorded on a 6545 LC-QTOF/MS system (Agilent Technologies) in the positive ion mode.

4.2. Materials

The freeze-dried water extract from dried roots (Radix) of *Eurycoma longifolia* Jack (Simaroubaceae) was kindly provided by Phytes Biotek Sdn Bhd (Malaysia).

4.3. Extraction and isolation

One hundred and five grams of the provided powder was subjected to fractionation with solvents of increasing polarity to give *n*-hexane-, dichloromethane- and ethyl acetate-soluble fractions. The weights of them were as following: 302 mg, 1.811 g, 1.843 g and 100 g. The *n*-hexane-soluble fraction was chromatographed on Buchi Reveleris® Prep system equipped with NP-silica gel set with UV-ELSD detector and using a gradient mobile phase of *n*-hexane/EtOAc (100 : 0 → 0 : 100). Fractions eluted with approximately *n*-hexane/EtOAc (40 : 60) was recovered as a pure compound **8** (3.2 mg) from this run. Fractions [eluted with *n*-hexane/EtOAc (70 : 30)] was chromatographed on Buchi Reveleris® Prep system equipped with a preparative Inertsil ODS-3 column (5 μm, Ø 20 × 250 mm, GL Sciences Inc., Japan) using H₂O/MeOH (60 : 40 → 0 : 100) to afford compounds **9** (20 mg), **7** (1 mg), **6** (1 mg) and **5** (0.8 mg) eluted with H₂O/MeOH (50 : 50 → 27 : 73 → 8 : 92 → 2 : 98), respectively. The DCM-soluble fraction was chromatographed on Buchi Reveleris® Prep system equipped with RP-C₁₈ silica set with UV-ELSD detector and using a gradient mobile phase of H₂O/MeOH (95 : 5 → 0 : 100) to give 11 fractions (D1–D11). Fractions D1 and D2 were proved to be pure compounds and assigned as **10** (20 mg) and **11** (10.8 mg). Fraction D5 was chromatographed on Buchi Reveleris® Prep system equipped with RP-C₁₈ silica set with UV-ELSD detector and using a gradient mobile phase of H₂O/MeOH (95 : 5 → 60 : 40) to afford compound **4** (4 mg). The EtOAc-soluble fraction was subjected to a silica flash column (FlashPure ID HP 20 μm particles, 12 g) and eluted with *n*-hexane/EtOAc (2 : 1, EtOAc) and EtOAc–MeOH (9 : 1 → 4 : 1 → MeOH) to give five fractions (Fr.1–Fr.5). The Fr.3 (77.3 mg) was separated by semi-preparative HPLC (GL Sciences Inc. Ø 20 × 250 mm, 5 μm particles; UV detector 205 nm, flow rate: 8 mL min^{−1}) with



MeCN/H₂O gradient (20 : 80 → 100 : 0) as a mobile phase to yield to 3 (21.3 mg, $t_R = 30$ min). The Fr.4 (70 mg) was separated by semi-preparative HPLC (GL Sciences Inc. Ø 20 × 250 mm, 5 µm particles; UV detector 205 nm, flow rate: 8 mL min⁻¹) with MeCN/H₂O gradient (5 : 95 → 40 : 60) to yield to 1 (5.1 mg, $t_R = 38$ min) and 2 (2.2 mg, $t_R = 41.6$ min).

4.4. Cell culture

Human neuroblastoma SH-SY5Y (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's medium (high glucose) (Wako, Osaka, Japan). The medium was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Corning, CA, USA) and antibiotics 100 U mL⁻¹ penicillin–streptomycin (Wako, Osaka, Japan). The cells were grown at 37 °C in 5% CO₂ humidified incubator. To estimate the expression of AANAT gene by real-time qPCR, SH-SY5Y cells (1.0×10^5 cells per mL) were cultured for 24 hours and then treated with TA or compounds for 24 hours.

4.5. RNA extraction and real-time qPCR

Total RNA was prepared from cells using PureLink RNA Mini kit (Invitrogen, MA, USA) following the manufacturer's instructions. cDNA was synthesized from the extracted total RNA by ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Real-Time quantitative PCR was performed by Agilent AriaMX real-time PCR system (Agilent Technologies, CA, USA). Using the THUNDERBIRD SYBR qPCRmix (TOYOBO, Osaka, Japan) for the real-time qPCR reaction. The real-time qPCR reaction conditions were initial denaturation at 95 °C for 60 s cDNA samples were amplified for 40 cycles (95 °C for 15 s and 60 °C for 60 s). Primers used for amplification were TGCCAGTGAGTTTCGCTGCCTC and GTCAGGAAGTCCCGATCTCAT for AANAT, or GTCTCCTCTGACTTCAACAGCG and ACCACCCTGTTGCTGTAGCCAA for GAPDH. The expression level of the AANAT gene was analyzed using $\Delta\Delta C_t$ analysis mode with AriaMx Real-Time PCR software.

Abbreviations

AANAT	Arylalkylamine <i>N</i> -acetyltransferase
TLC	Thin layer chromatography
HPLC	High-performance liquid chromatography
NMR	Nuclear magnetic resonance
HRMS	High resolution mass spectrometry
PCR	Polymerase chain reaction

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions

Isolation & Structure elucidation of quassinoids: C. Han, L. Meng. Isolation of other known secondary metabolites: Y. Amen

and M. Elsbaey. Writing original draft: C. Han. Evaluation of Activity: M. Nagata and M. Matsumoto. Funding acquisition: Y. Kuroki. Project administration: Kuniyoshi Shimizu.

Conflicts of interest

The authors have declared no conflicts of interest.

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