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Sample pre-treatment strategy for value-added bioactive phytochemicals in adventitious root culture of *Eurycoma harmandiana* Pierre and evaluation of their anti-inflammatory activity

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ABSTRACT: *Eurycoma harmandiana* Pierre (Simaroubaceae) (EH) is a well-known herbal plant with aphrodisiac and antifever properties. This study investigated the effect of a pre-treatment strategy to enhance aglycone metabolites in the adventitious root culture of EH and evaluated its anti-inflammatory activity. The content of pre-treatment samples was determined using HPLC analysis. Nitric oxide (NO) production was evaluated using LPS-stimulated RAW 264.7 macrophage cells. The anti-inflammatory activity of the pre-treatment (Tx) and non-treatment (non-Tx) samples was compared using quantitative polymerase chain reaction of inflammatory genes, including inducible NO synthase (iNOS), tumor necrosis factor-α (*TNF-*α), cyclooxygenase-2 (*COX-2*), interleukin-1β (*IL-1*β), and interleukin-6 (*IL-6*). Deglycosylation of canthin-6-one-9-O-β-glucopyranoside (C6OG) to 9-hydroxy-canthin-6-one (HCO) occurred in the Tx group by pre-treatment method. The enrichment of the aglycone sample exhibited a level of HCO (aglycone form) that was significantly higher (9.3-fold) than that of the non-Tx. Moreover, HCO had superior anti-inflammatory activity than the glycoside form. The enriched aglycone sample had higher potency than the non-Tx and suppressed inflammatory gene expression. Our findings provide novel insights into the benefits of using the pre-treatment method in health supplement products.

KEYWORDS: *Eurycoma harmandiana*, sample pre-treatment, canthin-6-one alkaloids, adventitious root culture, anti-inflammatory activity

INTRODUCTION

Eurycoma harmandiana (EH), a herbal plant belonging to the Simaroubaceae family, was discovered in northeast Thailand. The root is in high demand as an aphrodisiac herbal supplement [1]. In Thai traditional medicine, EH root is used as an ingredient in the Chantaleela remedy, indicated for treating fevers and common cold symptoms. EH root and its chemical components are active against inflammation [2], malaria [3], SARS-CoV-2 infection [4], and erectile dysfunction [5, 6]. Owing to high consumption rates, EH plants are endangered because they need a long time to re-grow. Plant tissue culture is a method to preserve endangered plants and used in the research field, especially in herbal plants [7]. Methods for improving plant growth and producing secondary metabolites are focused on important medicinal plants or endangered plants [8]. In a previous study, callus and adventitious root cultures of EH were established, and their phytochemical content and anti-inflammatory activity were evaluated in RAW 264.7 macrophage cells [9].

The inflammatory process is an immune response mechanism that protects the human body from invaders and maintains homeostasis. When humans encounter pathogens or inflammatory stimuli, macrophage cells are activated and regulate a defensive signaling pathway to initiate the inflammatory response [10]. On the surface of macrophages, Toll-like receptor 4 (TLR4) is present and binds to the invader such as lipopolysaccharide (LPS). This binding triggers signaling cascades, leading to the activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [11]. Subsequently, NF-κB moves into the nucleus, where it enhances the transcription of genes responsible for producing pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [12], chemokines, and enzymes that play a crucial role in the inflammatory process, including iNOS and COX-2 [13].

Sample pre-treatment, performed before the plant dries, involves grinding the plant to a paste and adding cellulase to the fresh sample to enhance the deglycosylation of active compounds in targeted plants. This method can be applied to fresh plants and *in vitro* plant cultures containing highly endogenous enzymes to produce enriched aglycone samples [14, 15]. Generally, the aglycone compound is an active form that exhibits pharmacological activity; the sugar-linked structure can be removed from the aglycone part by

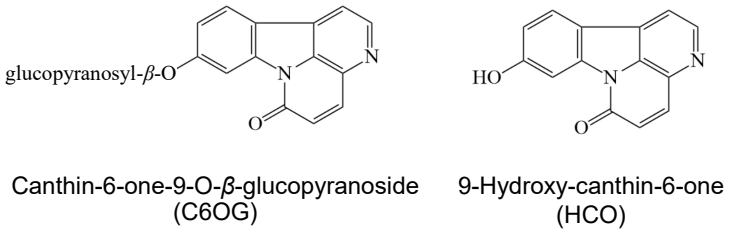

Fig. 1 Chemical structures of canthin-6-one-9-O-β-glucopyranoside (C6OG) and 9-hydroxy-canthin-6-one (HCO).

enzymatic hydrolysis [16]. For example, quercetin, an aglycone deglycosylated from rutin (quercetin-3-rutinoside), has improved antioxidant activity [17]. The hydrolysis of aucubin, a major iridoid glucoside in *Plantago* species, by plant-derived β-glucosidase enzyme showed interaction related to plant defensive system [18].

Canthin-6-one (canthinone) and derivative alkaloids, which are categorized to β-carboline alkaloids, are commonly found in the bark and root of Simaroubaceae, Rutaceae, Malvaceae, and Amaranthaceae plants [19]. The intact root of EH showed highly accumulated canthin-6-one alkaloids, mainly 9-hydroxy-canthin-6-one (HCO), the aglycone, with canthin-6-one-9-O-β-glucopyranoside (C6OG) as the glycoside (Fig. 1). Previous studies reported the major accumulation of canthin-6-one alkaloids in the roots of *Eurycoma* plants (approximately 0.1–0.4% w/w of crude residue) such as 9-methoxycanthin-6-one (0.2% w/w), 9-hydroxycanthin-6-one (0.2–0.4% w/w), and canthin-6-one 9-O-β-D-glucopyranoside (0.04% w/w) [20, 21]. The canthin-6-one alkaloids, including HCO and C6OG, exhibit anti-inflammatory activity and inhibit phosphodiesterase-5 for alleviating erectile dysfunction [5, 22, 23]. Previous studies demonstrated that plant tissue culture techniques such as callus and adventitious root culture from EH resulted in the accumulation of canthin-6-one alkaloids, especially C6OG [5, 9]. Pre-treatment methods involving the deglycosylation reaction of bioactive compounds may help increase the aglycone component which values the raw materials and can be applied to agricultural fields.

Therefore, this study observed the effect of a pre-treatment strategy to enhance aglycone metabolites in the adventitious root culture of EH. In addition, the anti-inflammatory activity of the pre-treatment sample was compared with that of the untreated sample. The findings from this study may provide insights into the development of alternative raw materials for plant and health-supplement products.

MATERIALS AND METHODS

Adventitious root culture of EH and plant source

The adventitious roots of EH have been established in a previous study [24]. The authentic plants and seeds were collected from Ubon Ratchathani, Thailand, and identified by Dr. Thaweesak Juengwatanatrakul, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University. An authentic specimen was submitted to the botanical herbarium of Pharmaceutical Sciences, Khon Kaen University (List number: NI-PSKKU 118). The adventitious roots were cultured in a half-strength Murashige and Skoog (MS) liquid medium containing 5 mg/l 1-naphthaleneacetic acid (NAA, Fluka Chemika, Buchs, Switzerland). The subculturing of adventitious roots was carried out by renewing the medium every 8 weeks. The 12^{th} subculture of fresh adventitious roots was harvested for a pre-treatment study and evaluated for anti-inflammatory activity.

Plant sample preparation and extraction

The adventitious roots of EH were harvested and divided into 2 groups: control (non-Tx) and sample pre-treatment (Tx). The non-Tx was processed by drying the fresh sample at 50 °C overnight. Subsequently, the dried sample was ground into powder and accurately weighed to 50 mg. Ultrasonic extraction was carried out for 20 min while absolute ethanol AR (0.5 ml) was added. By centrifuging the supernatant at 4,300×g for 10 min, the supernatant was separated. Four repetitions of extraction were done, and the extract solution was dried at 25 °C. Absolute ethanol AR (1 ml) was added to the crude extract to achieve the final concentration (mg dry weight/ml). Tx was performed by grinding fresh adventitious roots in a mortar into a paste. Then, the paste was dried at 50 °C overnight. The dried paste was ground into a powder, and the extraction process was performed in the same manner as for the non-Tx group.

Chromatographic conditions

HPLC analysis and reference compounds were isolated and performed as previously described [24]. Briefly, a gradient flow was set at 0.02% trifluoroacetic acid in water (A) and acetonitrile (B): 8% to 80% in B (0–30 min) at a rate of 1.0 ml/min, and the detector was selected at 244 nm. A SHIMADZU i-series (Japan) and reversed-phase column (Merck LiChrospher 100, C18, Germany) were used in the experiment.

Evaluation of anti-inflammatory activity using RAW 264.7 macrophage cells

The RAW 264.7 macrophage cells (Cell Line Service, Eppelheim, Germany) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco, CA, USA) supplement with 10% (v/v) fetal bovine serum (FBS, Gibco) and 100 units/ml penicillin/streptomycin (Gibco) under 5% CO_2 at 37 °C. Cultured cells with density 2×10^5 cells/ml (2×10^4 cells/well) were seeded in 96-well plates for the experiment. After 24 h, the selected extract was applied to the cells in a variety of concentrations, and they were stimulated for 24 h either with or without 0.03 µg/ml bacterial lipopolysaccharide (LPS, Sigma-Aldrich, MO, USA). The extracts were dissolved in DMSO and then diluted in culture medium with keeping the final DMSO concentration below 0.1%. Following the test, the medium was taken for the NO assay, and the cells were taken for cell viability evaluations. NO was measured using the Griess reagent and detected at 550 nm, compared to a standard curve of sodium nitrite (working range: 0.78–2.5 µg/ml) [25]. The inhibition of NO production was calculated using the following equation:

$$\%\ \text{NO inhibition} = \frac{\text{Nitrite}_{\text{cell control}} - \text{Nitrite}_{\text{sample treatment}}}{\text{Nitrite}_{\text{cell control}}} \times 100$$

LPS-treated cells (cell control) were set to fully NO production, and the positive control was N(γ)-nitro-L-arginine methyl ester (L-NAME, Sigma-Aldrich), which was used as a NO inhibitor via the inflammatory process in this experiment.

Cell viability assay

The harvested cells were examined using a 0.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) solution in DMEM media. The reagent was incubated for 2 h, and the precipitate was dissolved in isopropanol AR. The optical density (OD) was determined at 570 nm using a microplate reader (Biochrom, USA) [26]. The percentage of cell viability was calculated using the following equation:

$$\%\ \text{Cell viability} = \frac{\text{OD}_{\text{sample treatment}}}{\text{OD}_{\text{cell control}}} \times 100$$

LPS-treated cells (cell control) were set to 100% cell viability, and L-NAME-treated cells were set as positive control.

Real-time PCR measurement of inflammatory gene expression

TRIzol reagent (Invitrogen™, CA, USA) was used to extract the harvested cells in accordance with the manufacturer's instructions. BioDrop DUO (USA) was used to measure the amount of RNA, and the yield of RNA was calculated as µg/ml. Following the manufacturer's instructions, first-strand cDNA was created using ReverTra Ace™ qPCR RT Kit (Toyobo, Japan). The expression levels of inflammatory genes were determined by real-time PCR using SYBR Green PCR Master Mix (Bio-Rad, CA, USA). The PCR conditions were optimized, the relative gene expression was calculated as previously described [24], and β-actin was chosen as the reference gene. The results are shown as fold expressions after analysis with Biorad-iQ5 software and calculated using the $2^{-\Delta\Delta Ct}$ method [27]. The primer sequences were given in Table S1.

Statistical analysis

Data ($n = 3$) were analyzed and reported as mean ± standard deviation (SD) by using Microsoft Excel 365. The comparison of phytochemical content between the Tx and non-Tx groups was performed using an independent t-test ($p < 0.05$). The level of NO and relative quantification of inflammatory genes were determined using one-way analysis of variance (ANOVA) with Dunnett's test (SPSS version 26, IBM Corp.).

RESULTS AND DISCUSSION

Effect of sample pre-treatment on secondary metabolites in adventitious roots of EH

The phytochemical content determination of pre-treatment (Tx) and control (non-Tx) EH adventitious roots were performed using HPLC. Quassinoids, β-carboline, and canthin-6-one alkaloids were used as the standard compounds in this study. The HPLC results are shown in Table 1, and the chromatograms of authentic compounds and samples are shown in Fig. 2. 13β,21-dihydroeurycomanone, chaparrinone, 7-hydroxy-β-carboline-1-propionic acid, and 7-methoxy-β-carboline-1-propionic acid were not detected in the adventitious root of EH. A comparison of secondary metabolite levels between the Tx (Fig. 2c) and non-Tx groups (Fig. 2b) showed that the level of HCO (aglycone form) in the Tx group was significantly higher (9.3-fold) than that in the non-Tx group ($p < 0.05$). The levels of eurycomalactone, β-carboline-1-propionic acid, and canthin-6-one were not significantly different between the 2 groups; however, the levels of 13-α(21)-epoxy-eurycomanone, eurycomanone, and 9-methoxy-canthin-6-one were lower in the Tx group than those in the non-Tx group. The level of C6OG (glycoside form) in the Tx group was lower than that in the non-Tx group, demonstrating the effect of endogenous enzymatic hydrolysis on the cleavage of the sugar linkage form C6OG from the aglycone form HCO.

The pre-treatment strategy for fresh plants is related to enzyme activity in plant cells, especially β-glucosidases, which play an important role in the

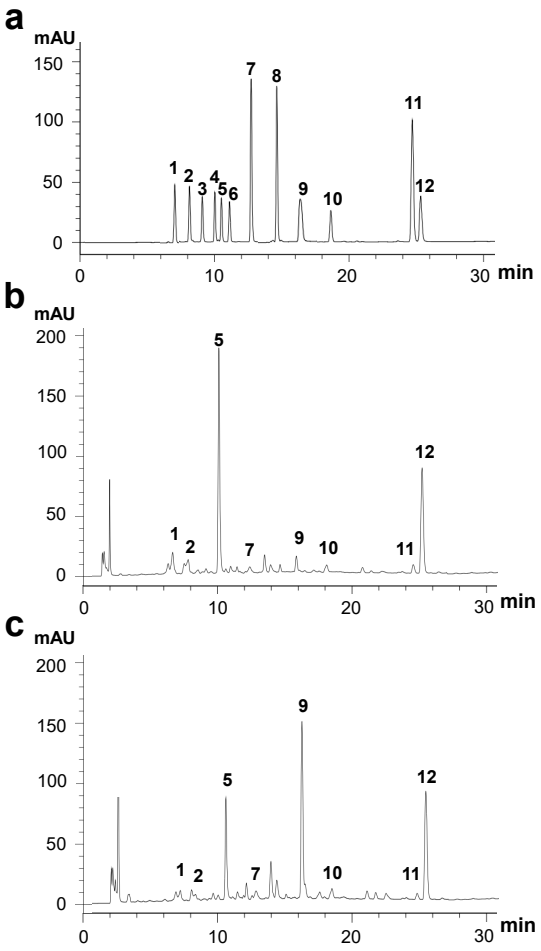

Fig. 2 HPLC chromatogram of (a) reference compounds, (b) adventitious root of EH (non-Tx), and (c) sample pre-treatment of the adventitious root of EH (Tx): 13-α(21)-epoxyeurycomanone (1), eurycomanone (2), 13β, 21-dihydroeurycomanone (3), 7-hydroxy-β-carboline 1-propionic acid (4), canthin-6-one-9-O-β-glucopyranoside (5), chaparrinone (6), β-carboline-1-propionic acid (7), 7-methoxy-β-carboline 1-propionic acid (8), 9-hydroxy-canthin-6-one (9), eurycomalactone (10), canthin-6-one (11), and 9-methoxy-canthin-6-one (12).

hydrolysis of the β-glucosidic bond between sugar and aglycone moieties. When the cell wall of a plant is destroyed, stress conditions would trigger defensive mechanisms and enzymatic activity. The β-glucosidase was found in the plant cell or expressed in microbial strains by using recombinant technique [28, 29]. The goal of biotransformation with β-glucosidases was to improve the yield of the aglycone compound, which related to increase pharmacological activity. Many studies have proven the effect of enzymatic hydrolysis from both endogenous and exogenous sources. For example, the enhancement of oxyresveratrol content in *Morus alba* callus by endogenous enzymatic hydrolysis on fresh callus [14], the biotransformation of ononin to formononetin in licorice callus with simple grounding to fresh callus enhancing their anti-inflammatory properties [15], the β-glucosidase effect to produce the cucurbitacin B content in *Cucumis melo* pedicel by biotransformation from its glycoside [30]. Besides, the β-glucosidase enzymes could be related to the activation of the defensive system by benzoxazinoid deglycosylation in *Lamium galeobdolon* leaves [31].

Effect of sample pre-treatment of adventitious root of EH on cell viability of RAW 264.7 macrophage cells

The various concentrations of C6OG (50–12.5μM), HCO (50–12.5 μM), and sample pre-treatment of adventitious root of EH (200–50 μg dry wt./ml) were used to screen cell viability on LPS-stimulated RAW macrophage cells. The percentage of cell viability with treated samples that reach 80% were implied to non-toxicity in treated cells. The data in Fig. 3 exhibited the percentage of cell viability on selected samples while the LPS (+) treated group was set as 100% cell viability. The positive group with L-NAME (250 μM) showed cell viability by 118.50 ± 4.90%. The concentrations of C6OG and HCO at 50–12.5 μM and the ethanolic extract of adventitious root of EH at 100–50 μg dry wt/ml showed non-toxicity on treated cells. Thus, the selected concentration with non-toxicity would be evaluated for anti-inflammatory activity and inflammation gene expression.

Effect of sample pre-treatment of adventitious root of EH on anti-inflammatory activity and inflammatory gene expression

NO inhibitory activity was assessed using LPS-stimulated RAW macrophages to evaluate the effect of sample pre-treatment on the anti-inflammatory potential of adventitious roots of EH. Various concentrations of marker compounds (C6OG and HCO) and extracts (Tx and non-Tx) were used to determine cell viability. The concentrations that showed cell viability greater than 80% (compared to the (+) LPS group) were selected and used for NO assay (Table 2). The positive control, L-NAME (250 μM), inhibited NO by 81.99 ± 1.85%. Comparing the NO inhibitory activity of canthin-6-one alkaloids, we found that C6OG and HCO showed a dose-dependent reduction in NO production, and the highest dose of HCO (50 μM) significantly inhibited NO compared with the glycoside form at the same concentration. In addition, Tx showed dose-dependent NO inhibition at non-toxic doses (100, 75, and 50 μg dry wt/ml). At concentration of 100 and 75 μg dry wt/ml, the Tx group showed significant NO inhibition compared to the non-Tx group at the same concentration.

In RAW macrophage cells treated with C6OG,

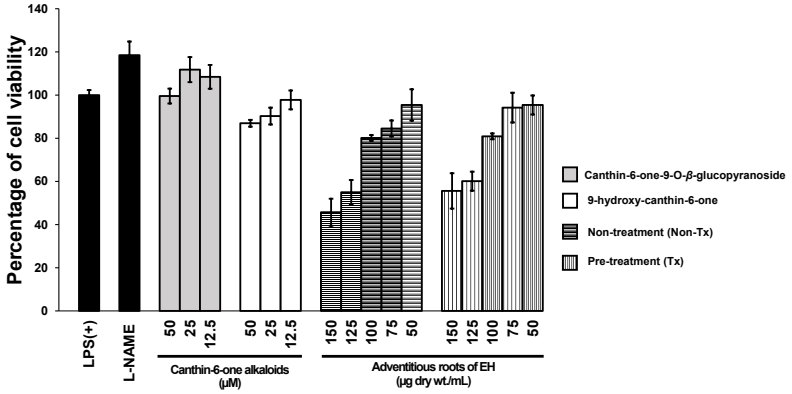

Fig. 3 Cell viability on LPS-stimulated RAW macrophages cells treated with canthin-6-one-9-O-β-glucopyranoside, 9-hydroxy-canthin-6-one, and adventitious root of EH.

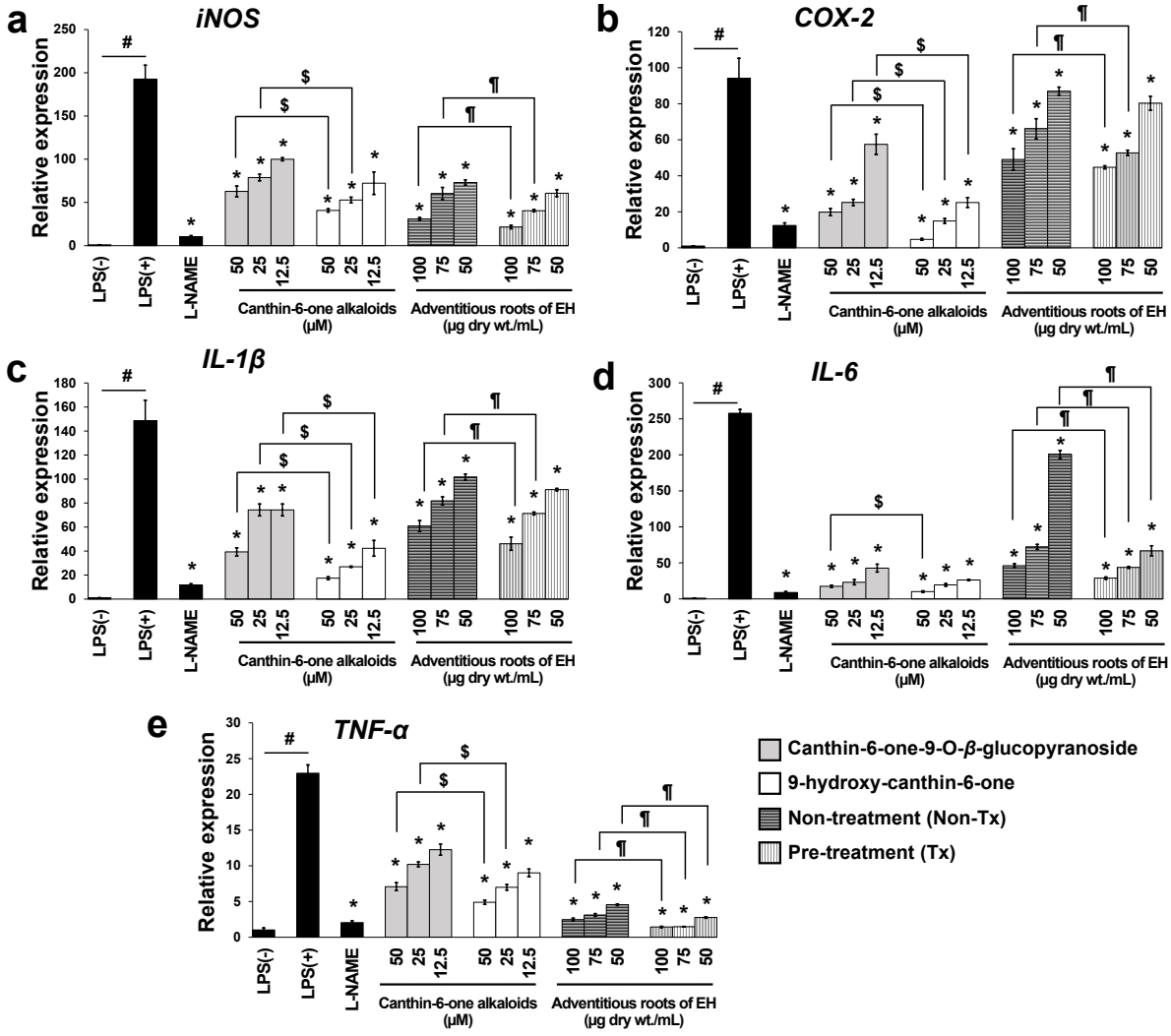

Fig. 4 Relative quantitation of inflammatory gene mRNAs in RAW 264.7 macrophage cells treated with canthin-6-one-9-O-β-glucopyranoside (C6OG), 9-hydroxy-canthin-6-one (HCO), and adventitious root of EH: *iNOS* (a), *COX-2* (b), *IL-1*β (c), *IL-6* (d), and *TNF*-α (e). $^{\#}$ Significant level ($p < 0.05$) compared with (-) LPS group, * significant level ($p < 0.05$) compared with (+) LPS group, $^{\$}$ significant level ($p < 0.05$) comparison between C6OG to HCO at the same concentration, and π the significant level ($p < 0.05$) comparison between Tx to non-Tx at the same concentration.

Table 1 Content of quassinoids, β-carboline alkaloids, and canthin-6-one alkaloids in the adventitious root culture of EH.

Class	Compound	Content (mg/g dry weight)	
		Non-Tx	Tx
Quassinoid	13-α(21)-epoxy-eurycomanone (1)	0.41 ± 0.01	0.12 ± 0.01*
	eurycomanone (2)	0.35 ± 0.00	0.12 ± 0.01*
	13β,21-dihydroeurycomanone (3)	ND	ND
	chaparrinone (6)	ND	ND
	eurycomalactone (10)	0.25 ± 0.00	0.25 ± 0.00
β-carboline alkaloid	7-hydroxy-β-carboline-1-propionic acid (4)	ND	ND
	β-carboline-1-propionic acid (7)	0.05 ± 0.00	0.06 ± 0.00
	7-methoxy-β-carboline-1-propionic acid (8)	ND	ND
Canthin-6-one alkaloid	canthin-6-one-9-O-β-glucopyranoside (5)	3.77 ± 0.18	1.39 ± 0.06*
	9-hydroxy-canthin-6-one (9)	0.19 ± 0.00	1.77 ± 0.00*
	canthin-6-one (11)	0.05 ± 0.00	0.02 ± 0.00
	9-methoxy-canthin-6-one (12)	2.03 ± 0.02	1.74 ± 0.02*

ND = not determined. * Significant level of comparison between sample pre-treatment (Tx) to control (non-Tx) with p value < 0.05 using independent t-test.

Table 2 Anti-inflammatory activity in RAW 264.7 macrophage cells treated with C6OG, HCO, and adventitious root of EH.

Sample	Concentration	Nitrile (μM)	NO inhibition (%)	Cell viability (%)
(-) LPS	–	2.56 ± 0.43	–	–
(+) LPS	0.03 μg/ml	26.34 ± 1.14#	–	100.00 ± 0.00
L-NAME	250 μM	04.65 ± 0.61*	81.99 ± 1.85*	118.50 ± 4.90
C6OG	50 μM	16.47 ± 0.47*	37.47 ± 1.78*	99.56 ± 3.43
	25 μM	20.15 ± 0.75*	23.50 ± 1.42*	111.79 ± 5.81
	12.5 μM	24.38 ± 0.73	07.44 ± 2.84	108.46 ± 5.51
HCO	50 μM	12.70 ± 0.31*,$	51.78 ± 2.31*,$	81.95 ± 0.56
	25 μM	19.97 ± 0.65*	24.18 ± 1.86*	90.29 ± 3.90
	12.5 μM	23.29 ± 0.57	11.58 ± 2.11	97.76 ± 4.35
Non-Tx	100 μg dry wt./ml	13.06 ± 0.82*	50.42 ± 3.54*	80.90 ± 1.35
	75 μg dry wt./ml	16.75 ± 0.52*	36.40 ± 2.21*	94.19 ± 6.89
	50 μg dry wt./ml	19.88 ± 0.83*	24.53 ± 4.16*	95.42 ± 4.40
Tx	100 μg dry wt./ml	11.75 ± 0.39*,π	55.39 ± 5.21*,π	80.13 ± 1.34
	75 μg dry wt./ml	13.79 ± 0.37*,π	48.03 ± 2.25*,π	84.52 ± 3.71
	50 μg dry wt./ml	16.65 ± 0.39*	36.79 ± 4.47*	95.42 ± 7.26

C6OG: canthin-6-one-9-O-β-glucopyranoside; HCO: 9-hydroxy-canthin-6-one; Non-Tx: non-treatment; and Tx: pre-treatment. Data represented as mean ± standard deviation (SD). # Significant level ($p < 0.05$) compared with (-) LPS group, * significant level ($p < 0.05$) compared with (+) LPS group, $ significant level ($p < 0.05$) comparison between C6OG to HCO at the same concentration, and π significant level ($p < 0.05$) comparison between Tx to non-Tx at the same concentration.

HCO, Tx, and non-Tx adventitious root extracts of EH, the expression levels of genes related to inflammation were assessed using real-time PCR. The changes in the levels of inflammation signalling molecules such as *iNOS*, *COX-2*, *IL-1*β, *IL-6*, and *TNF-*α were evaluated. Inflammatory mediator gene expression was reduced by Tx and non-Tx extracts in response to LPS stimulant. The effectiveness of HCO (aglycone form) versus C6OG (glycoside form) in modifying inflammatory gene expression is shown in Fig. 4. The levels of inflammatory genes, including *iNOS* (Fig. 4a), *COX-2* (Fig. 4b), *IL-1*β (Fig. 4c), *IL-6* (Fig. 4d), and *TNF-*α (Fig. 4e), were significantly decreased by both compounds in a dose-dependent manner. When compared with C6OG at the same concentration, HCO showed a greater decrease in the expression of all inflammatory genes at a concentration of 50 μM. This suggests that the anti-inflammatory activity of the aglycone form is stronger than that of the glycoside form. The evaluation of the Tx and non-Tx extracts revealed that all chosen doses were able to inhibit all inflammatory genes in a dose-dependent manner. The highest concentration of Tx extract (100 μg dry wt/ml) significantly reduced the expression of all inflammatory genes when compared

to non-Tx extract at the same concentration.For each inflammatory gene, the IL-6 gene expression (Fig. 4d) was more effectively suppressed by the Tx extract than by the non-Tx extract. This suppression could be attributed to HCO, which leads to stronger anti-inflammatory activity among all the derivatives of canthin-6-one [9].

Our findings indicated that pre-treatment of the adventitious root culture of EH produced enriched aglycone canthin-6-one in the Tx group. It can be explained by endogenous enzyme activity in plant cells that activated hydrolysis reactions to produce an aglycone compound from glycoside residue. Further, anti-inflammatory activity was assessed to evaluate the effect of the high content of HCO on Tx compared with the non-Tx group. It seems to correlate with aglycone content and show anti-inflammatory activity in the same manner as Tx group.

The bacterial cell wall contains LPS that can bind to toll-like receptor 4 (TLR4) and induce inflammatory mediators such as *TNF-α*, *COX-2*, *IL-1β*, and *IL-6*. LPS was used to induce inflammation in RAW 264.7 macrophage cells. In this study, the *in vitro* anti-inflammatory model by determination of NO inhibition in RAW 264.7 macrophage cells were assessed. A lower level of NO production indicates a higher anti-inflammatory activity [32, 33].

The pre-treatment strategy produced enriched aglycone fraction by the deglycosylation of glycosides to aglycones; this aglycone demonstrated enhanced systemic absorption *in vivo* and better pharmacological activities such as anti-inflammatory and anti-cancer activities [34, 35]. In an *in vitro* anti-inflammatory model, the aglycone form exhibited greater activity than its glycosides. For example, formononetin exhibits higher activity than the glycoside form [34], and flavonol aglycones in green tea had superior anti-inflammatory activity than flavonol glycosides via suppression of *iNOS*, *IL-1β*, and *IL-6* gene expression [37].

Intact roots and callus cultures of EH were previously reported to have anti-inflammatory effects on RAW macrophages via suppression of *iNOS*, *COX-2*, and *IL-6* mRNAs as well as chemical constituents such as eurycomanoe, chaparrinone, and canthin-6-one alkaloids [9]. C6OG and HCO, found in the roots of EH and *Eurycoma longifolia*, exhibited NO inhibition in RAW macrophages. Additionally, HCO, produced from the hairy root culture of *E. longifolia*, exhibited *IL-6* and *TNF-α* suppression [22], Our findings on the anti-inflammatory activity of the adventitious root culture of EH were correlated with its native plant, as previously reported [9], and their inflammatory gene mRNA expression (*iNOS*, *COX-2*, *IL-1β*, *IL-6*, and *TNF-α*) was suppressed by a high content of canthin-6-one alkaloids, especially the enriched aglycone sample, by the pre-treatment method.

CONCLUSION

This study presented the effect of pre-treatment on fresh *in vitro* adventitious roots of EH that produced enriched aglycone samples by endogenous enzymatic hydrolysis. The deglycosylation of glycosides into aglycones in the sample treatment can enhance the anti-inflammatory activity via the RAW 264.7 macrophage model and suppress inflammatory genes. Our findings indicate a strategy that could be applied to increase beneficial bioactive metabolites and produce high-value raw materials for health supplement products.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at https://dx.doi.org/10.2306/scienceasia1513-1874.2024.084.

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Appendix A. Supplementary data

Table S1 The primer used in this study.

Gene	Primer
*β-actin*_F:	5′-GACAGCAGTTGGTTGGAGCA-3′
*β-actin*_R:	5′-GCGACCATCCTCCTCTTAGG-3′
*iNOS*_F:	5′-GCTATGGCCGCTTTGATGTG-3′
*iNOS*_R:	5′-ACCTCCAGTAGCATGTTGGC-3′
*IL-6*_F:	5′-TGGAGTCACAGAAGGAGTGGCTAAG-3′
*IL-6*_R:	5′-TCTGACCACAGTGAGGAATGTCCAC-3′
*COX-2*_F:	5′-CCTGCTGCCCGACACCTTCA-3′
*COX-2*_R:	5′-AGCAACCCGGCCAGCAATCT-3′
*IL-1β*_F:	5′-GCCTTGGGCCTCAAAGGAAAGAATC-3′
*IL-1β*_R:	5′-GGAAGACACCGATTCCATGGTGAAG-3′
*TNF-α*_F:	5′-ATAGCTCCCAGAAAAGCAAGC-3′
*TNF-α*_R:	5′-CACCCCGAAGTTCAGTAGACA-3′