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Production of Canthin-6-One and Stigmasterol in *Eurycoma longifolia* (Tongkat Ali) Jack. Hairy Root Culture Transformed using *Agrobacterium rhizogenes* Strain A4

(Pengeluaran Canthin-6-One dan Stigmasterol dalam *Eurycoma longifolia* (Tongkat Ali) Jack. Kultur Akar Rerambut Diubah menggunakan *Agrobacterium rhizogenes* Strain A4)

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ABSTRACT

Eurycoma longifolia, commonly known as Tongkat Ali or Malaysian ginseng, contains a variety of chemical compounds, including quassinoids, canthin-6-one alkaloids, B-carboline alkaloids, squalene derivatives, and steroids. These compounds contribute to the plant's therapeutic properties, which include anti-cancer, anti-malarial, anti-ulcer, aphrodisiac, and energy-boosting effects. Since many of these compounds are primarily extracted from the roots, overharvesting can threaten wild populations of *E. longifolia*. A sustainable approach to producing these chemical compounds involves cultivating hairy root cultures, which have been used successfully to produce chemical compounds in other plant species. In this study, hairy root cultures of *E. longifolia* were established using the *Agrobacterium rhizogenes* strain A4. The hairy roots were cultured in MS basal liquid medium at pH 4.9, under dark conditions on an orbital shaker (150 rpm) at 25 ± 2 °C. The production of two chemical compounds reported extracted from *E. longifolia* natural roots, canthin-6-one and stigmasterol, was confirmed in the extracts of the *E. longifolia* transformed hairy roots through High-Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopy analysis. *E. longifolia* hairy roots culture can be used as an alternative source for these chemical compounds.

Keywords: *Agrobacterium rhizogenes;* chemical compounds; hairy roots; Tongkat Ali

ABSTRAK

Eurycoma longifolia yang dikenali sebagai Tongkat Ali atau ginseng Malaysia mengandungi pelbagai sebatian kimia, termasuk quassinoid, alkaloid canthin-6-one, alkaloid B-carboline, terbitan squalene dan steroid. Sebatian ini menyumbang kepada sifat terapeutik tumbuhan ini, termasuk kesan anti-kanser, anti-malaria, anti-ulser, afrodisiak dan peningkatan tenaga. Oleh kerana kesemua sebatian ini diekstrak terutamanya daripada akar, pengambilan secara berlebihan boleh mengancam populasi liar *E. longifolia*. Pendekatan yang mampan untuk menghasilkan sebatian kimia ini melibatkan penghasilan kultur akar rerambut yang telah berjaya digunakan untuk menghasilkan sebatian kimia dalam spesies tumbuhan lain. Dalam kajian ini, kultur akar rerambut *E. longifolia* telah dibangunkan menggunakan strain *Agrobacterium rhizogenes* A4. Kultur akar rerambut tersebut dikulturkan ke dalam medium cecair asas MS dengan pH 4.9, di bawah keadaan gelap atas penggoncang orbital (150 rpm) pada suhu 25 ± 2 °C. Penghasilan dua sebatian kimia yang dilaporkan diekstrak daripada akar semula jadi *E. longifolia* iaitu canthin-6-one dan stigmasterol, telah disahkan dalam ekstrak akar rerambut *E. longifolia* menggunakan Kromatografi Cecair Berprestasi Tinggi (HPLC) dan analisis spektroskopi Resonans Magnetik Nuklear (NMR). Kultur akar rerambut *E. longifolia* boleh digunakan sebagai sumber alternatif untuk penghasilan dua sebatian kimia ini.

Kata kunci: *Agrobacterium rhizogenes;* akar rerambut; metabolit sekunder; Tongkat Ali

INTRODUCTION

Eurycoma longifolia, a member of the Simaroubaceae family known as Tongkat Ali, is a slow-growing tree in Southeast Asia such as Malaysia, Thailand, Indonesia, and Vietnam (Burkill 1966). Also known as Malaysian ginseng, it is considered as an adaptogen with male health-preserving effects (Tambi & Kadir 2006). *E. longifolia* roots are reported to contain different classes of bioactive compounds such as quassinoids, canthin-6-one alkaloids, B-carboline alkaloids, squalene derivatives, and bioactive steroids (Rehman, Choe & Yoo 2016). Quassinoids and alkaloids are the main constituent of *E. longifolia* compounds (Nazirah et al. 2018). These compounds have contributed to several bioactivities of *E. longifolia*, including antimalarial (Chan et al. 1986), antiulcer (Tada et al. 1991), cytotoxic effects (Kardono et al. 1991), and

aphrodisiac properties (Ang & Sim 1997). Since the active ingredients are mainly extracted from the roots, their use exposes the natural populations of *E. longifolia* to great harm.

Without utilising actual plants, several biotechnological tools have been utilised to cultivate plant tissues, organs, or undifferentiated cells in order to get useful plant metabolites (Alfermann & Petersen 1995; Yeoman & Yeoman 1996). By inserting Ti or Ri T-DNA into the genome of various plants, especially dicotyledons plants, utilising *Agrobacterium tumefaciens* and *A. rhizogenes*, plant transformation has been successfully attempted in numerous species (Burchi et al. 1996; Chilton et al. 1982). *A. rhizogenes* infection of damaged plant tissues led to the emergence of hairy roots with distinctive properties, including rapid growth rates independent of the presence of phytohormones in the growing media, limitless branching, and genetic and biochemical stability. Hairy roots have been used for the commercial-scale synthesis of chemical compounds in some species because they can produce chemical compounds that are similar to those produced by normal or wild roots, sometimes with higher production levels (Sharma, Padh & Shrivastava 2013). It has been a desirable option to induce hairy roots using *Agrobacterium*-mediated transformation. Hairy roots are stable genetically, and capable of producing a large number of metabolites that are often found in roots and other organs (Oksman-Caldentey & Hiltunen 1996). Numerous successful transformations have had significant commercial uses up to this point, particularly in the creation of chemical compounds with therapeutic value such as in species *Artemisia annua* for the production of artemisinin and *Azadirachta indica* A. Juss. for azadirachtin (Giri & Narasu 2000; Tripathi & Tripathi 2003).

Genetic transformation of *E. longifolia* using different strain of *A. rhizogenes* in an attempt to produce hairy roots culture has been carried out (Danial et al. 2012; Ngoc et al. 2015). Danial et al. (2012) reported that hairy roots culture of *E. longifolia* was successfully developed using wild type *A. rhizogenes* MAFF 210265, 301726 and 720002 on hypocotyl explants but phytochemical analysis on hairy roots produced were not reported. Meanwhile, Ngoc et al. (2015) utilized the *A. rhizogenes* strain ATCC 15834 to infect cotyledons and hypocotyl explants of *E. longifolia* resulting in the induction of hairy root growth and the identification of 9-methoxychanthin-6-one, 9-hydroxycanthin-6-one and β-carboline alkaloid namely 7-methoxy-(9H-β-carbolin-1-il)-(*E*)-1-propenoic acid derived from the hairy-root cultures of *E. longifolia.* At present, no reports yet on the production of canthin-6-one and stigmasterol from *E. longifolia* hairy roots. The objective of this study was to transform *E. longifolia* target tissue with *A. rhizogenes* strain A4 and to determine the production of the chemical compounds in the hairy root culture using High Performance Liquid Chromatography (HPLC) and Nuclear Magnetic Resonance (NMR) spectroscopy.

MATERIALS AND METHODS

PLANT MATERIALS AND CULTURE MEDIA

Seeds of *E. longifolia* collected from FRIM field station Maran were immersed in 70% ethanol with a drop of Tween 20 for 3 min before rinsing five times with sterile distilled water. Then, the seeds were washed with 50% Clorox® plus a drop of Tween 20 for 15 min followed by rinsing five times with sterile distilled water. Seed coats were removed and cotyledons were cultured on Murashige–Skoog (MS) (Murashige & Skoog 1962) basal medium containing 0.5 mg/L 6-Benzylaminopurine (BAP) and 0.8% (w/v) agar for *in vitro* germination. Media were autoclaved at 121 °C for 15 min and their pH was adjusted to 5.7-5.8 prior to the addition of plant growth regulator. All cultures were incubated at 25 °C in the 16/8-h light condition. The germinated plantlets were used as source of explants. Stems, petioles, roots and leaf segments from germinated plantlets were excised and used as target tissues for transformation experiments.

GENETIC TRANSFORMATION OF *E. longifolia* EXPLANTS

In this transformation experiment, freshly cut or 1-week pre-cultured *E. longifolia* explants (stems, petioles, roots and leaves) from *in vitro E. longifolia* germinated plantlets on MS basal media containing 1 mg/L NAA were used as target tissues. Meanwhile, *A. rhizogenes* strain A4 were cultured in 10 mL Luria-Bertani (LB) liquid medium supplemented with 5 mM glucose, 100 µg/mL rifampicin and 500 µg/mL streptomycin. The bacterial culture was maintained at 28 °C on shaker (200 rpm) for 12-16 h until an $O.D._{600}$ of 0.4 - 0.5 is achieved. 1 mL of the bacterial culture was then diluted with 35 mL of the same medium and used as bacterial suspension to infect the target tissues.

Infection of target tissues was carried out by dipping the explants in bacterial suspension for 1 h. The explants were then blotted dry using tissue paper and cultured on basal MS media. Co-cultivation was carried out for 3 days before target tissues were transferred onto basal MS media containing 250 µg/mL cefotaxime to remove the excess bacteria. All cultures were incubated at 25 °C in the growth room. About 3-4 weeks after inoculation, hairy root appeared from stem, root and petiole explants but none from leaves tissues. Only hairy roots from roots explants can be multiplied in basal MS medium. The hairy roots were subsequently cultivated on basal MS media supplemented with 250 µg/mL cefotaxime for multiple sub-cultures until bacteria-free hairy root cultures were achieved. Aseptic hairy root cultures were then transferred onto solid basal MS media and used as stock cultures for further multiplication and experiments.

ESTABLISHMENT OF *E. longifolia* HAIRY ROOT CULTURES IN LIQUID MEDIUM

Hairy roots from basal MS solid medium were cut into 1 cm length and 0.1 g hairy roots were inoculated into 50 mL basal MS liquid medium free from plant growth regulator. Cultures of hairy roots were shaking at 150 rpm in culture room with a temperature of 25 °C in the dark condition.

DNA ANALYSIS OF TRANSFORM HAIRY ROOTS

Genomic DNA of *E. longifolia* hairy roots were extracted using DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The purity and quality of extracted DNA was assessed by NanoDrop™ 2000 spectrophotometer (Thermo Scientific™, USA) and agarose gel electrophoresis. The PCR analysis was performed in a SimpliAmp™ thermal cycler (Applied Biosystems™, USA). The reaction mixture consisted of 7.5 µL of exTEN 2X PCR master mix (1st BASE, Singapore), 0.2 µm of each forward and reverse primers and 10 ng of DNA template in a final volume of 15 µL reaction. The PCR condition were as follows; initial denaturation at 95 °C for 4 min, then 30 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 45 s, elongation 72 °C for 30 s and a final elongation at 72 °C for 3 min. The PCR products were analysed in 2% (w/v) agarose gel electrophoresis.

CHEMICAL COMPOUNDS EXTRACTION AND ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) AND NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

E. longifolia hairy roots from flasks were harvested and dried overnight at 37 °C and then used for extraction with methanol. The dried hairy roots were extracted with methanol and concentrated using rotary evaporator. The dried extracts and standard compound solution of canthin-6-one and stigmasterol were dissolved in HPLC grade methanol and the solution was filtered through a membrane filter (pore size 0.45 µm) prior to HPLC analysis using a Waters HPLC system (Waters Delta 600 with 600 Controller) with photodiode array detector (Waters 996). A Phenomenex-Luna (5 µm) column was used (4.6 mm i.d. × 250 mm) and for elution of the constituents, a gradient of two solvents denoted as A and B was employed. A was 0.1% aqueous formic acid, whereas B was acetonitrile. Initial conditions were 35% A and 65% B, with a linear gradient reaching 45% A at t = 1 min. This was followed by an isocratic elution until t = 10 min, after which the programme returned to the initial solvent composition at t = 15 min and continued for 10 min. The column temperature was maintained at 40 °C. The flow rate used was 1.0 mL/min and the injection volume was 10 µl. The retention time and UV spectrum of major peaks were analysed. The structures of the compounds were elucidated using proton (^{1}H) and carbon (^{13}C) NMR spectroscopy on a Bruker DRX 300 NMR spectrometer (300 MHz for ^{1}H-NMR and 75 MHz for ^{13}C-NMR, in $CDCl_3$), and further confirmed by comparing the data with literature values.

RESULTS AND DISCUSSION

ESTABLISHMENT OF *E. longifolia* HAIRY ROOT CULTURES

Hairy roots appeared on the cut edges of stem, petiole and root explants three weeks after inoculation. But only hairy roots from root explants can grow rapidly in solid

TABLE 1. Primer used for PCR analysis of *E. longifolia* hairy roots

Primer name	Sequence (5'- 3')	Expected size of PCR product	References
rolA1-F	CTAAGATCATGCTGTAACGCTTC	~ 423 bp	Van der Salm et al. (1998)
rolA1-R	GCGTATTAATCCCGTAGGTTTGT		
rolB-F	CTTATGACAAACTCATAGATAAAGGTTG	~ 858 bp	Kim et al. (2007)
rolB-R	TCGTAACTATCCAACTCACATCAC		
rolC-F	ATGGCTGAAGACGACCTGTGTT	~ 543 bp	Mishiba et al. (2006)
rolC-R	TTAGCCGATTGCAAACTTGCAC		
rolD-F	CCTTACGAATTCTCTTAGCGGCACC	~ 477 bp	Kang et al. (2006)
rolD-F	GAGGTACACTGGACTGAATCTGCAC		
virC-F	ATCATTTGTAGCGACT	~ 730 bp	Kim et al. (2007)
virC-R	AGCTCAAACCTGCTTC		
virD1-F	ATGTCGCAAGGACGTAAGCCCA	~ 338 bp	Chávez-Vela, Chávez-Ortiz & Pérez-Molphe Balch (2003)
virD1-R	GGAGTCTTTCAGCATGGAGCAA		

and liquid growth regulator free MS medium (Figure 1). Hairy root cultured in this medium were used throughout this study. Even though there are reports by Danial et al. (2012) and Ngoc et al. (2015) on the genetic transformation of *E. longifolia* but they were using different strain of *A. rhizogenes.* The capacity of different strains of *A. rhizogenes* to successfully infect and alter the target plant varies (Balakrishnan et al. 2012). Morteza, Seyed and Masoud (2008) discovered strain and species specificity in the hairy root induction of four *Hyoscyamus* species using five different *A. rhizogenes* strains. Similarly, in *Torenia fournieri* genetic transformation, the strain with the highest root induction had a transformation frequency of 65%, while the strain with the lowest was 28.3% (Tao & Li 2006). When Alpizar et al. (2006) evaluated the transformation effectiveness of five *A. rhizogenes* strains on two species of coffee plants, they discovered that A4RS, an agropine type strain, was 80% more virulent than the other strains. The plasmids carried by bacterial strains could explain the difference in virulence (Nguyen et al. 1992), and the presence of *rol A*, *B*, and *C* genes was shown to be sufficient for developing the hairy root phenotype (Christey 2001).

PCR CONFIRMATION OF TRANSGENIC HAIRY ROOTS

The integration of *A. rhizogenes* T-DNA into the *E. longifolia* genome is verified by PCR amplification of plant oncogenes: *rolA1, rolB, rolC* and *rolD.* These genes were crucial for the formation of hairy roots (Boulanger, Berkaloff & Richaud 1986; Cardarelli et al. 1987; Moore, Warren & Strobel 1979) and the induction of the chemical compounds (Bulgakov 2008; Shkryl et al. 2008). Meanwhile, the absence of *Agrobacterium* contamination was further confirmed with virulence genes; *virC* and *virD1*. *Vir* genes which can be grouped into 8 operons (*virA, virB, virC, virD, virE, virF, virG,* and *virH*) are responsible for facilitating the transfer of T-DNA region but do not integrate into the plant genome (Sheng & Citovsky 1996; Shreni Agrawal 2022). Figure 2(A)-2(D) shows the presence of a single band for *rolA1, rolB, rolC,* and *rolD* with expected sizes of approximately 423 bp, 858 bp, 543 bp, and 477 bp, respectively, in *A. rhizogenes* (positive control) and transformed *E. longifolia* hairy roots. The *virC* and *virD1* genes were only found in *A. rhizogenes* whilst no bands were detected in the transformed *E. longifolia* hairy roots confirming that the sample was free from *A. rhizogenes* contamination (Figure 2(E)-2(F)).

HPLC AND NMR ANALYSIS

HPLC chromatogram and UV spectrum of canthin-6-one was shown in Figure 3. Stigmasterol cannot be detected using UV detector and for future study, the evaporative light scattering detection can be use. Meanwhile, NMR analysis showed that other than 9-metoxycanthin-6-one as reported by Nazirah et al. (2018) and Yunos et al. (2022)

(a) (b)

FIGURE 1. *E. longifolia* hairy root culture grown in: (a) solid basal MS medium, and (b) liquid basal MS medium

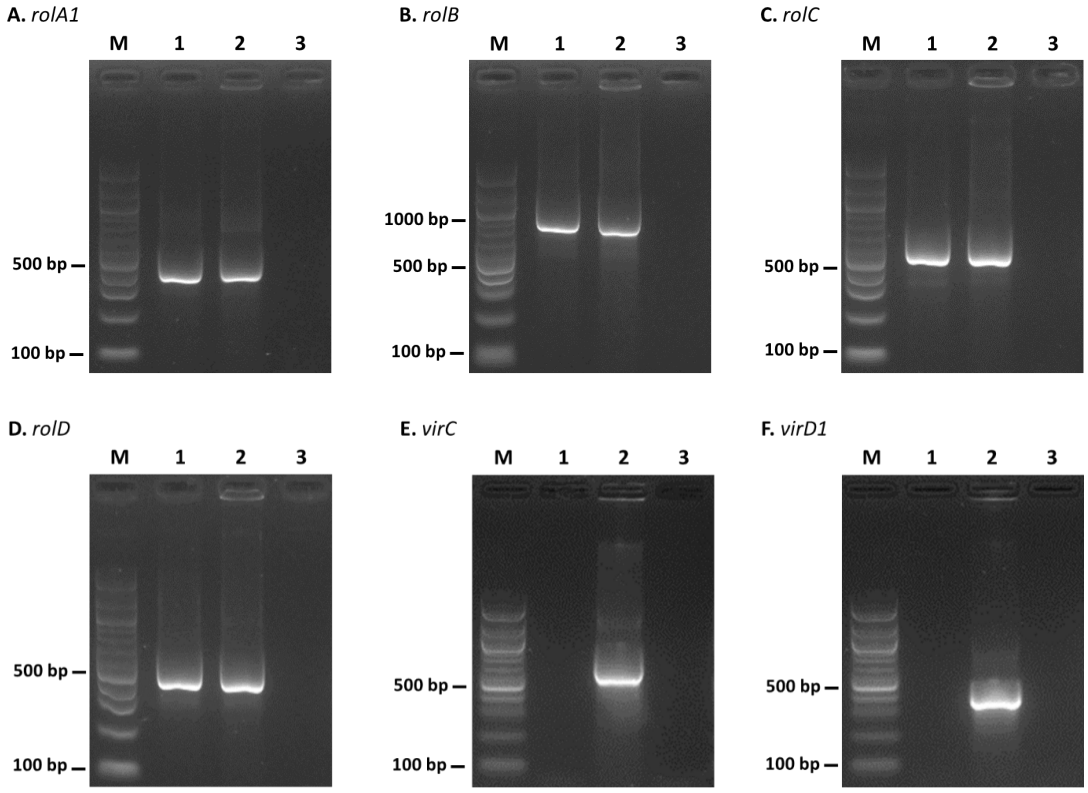

FIGURE 2. PCR analysis of *rolA1, rolB, rolC, virC,* and *virD1* genes in transformed *E. longifolia* hairy roots. Lanes: *M:* NEB 100 bp DNA ladder; *1:* DNA isolated from the hairy roots of transformed line; *2:* DNA isolated from *A. rhizogenes* as a positive control and *3:* non template control

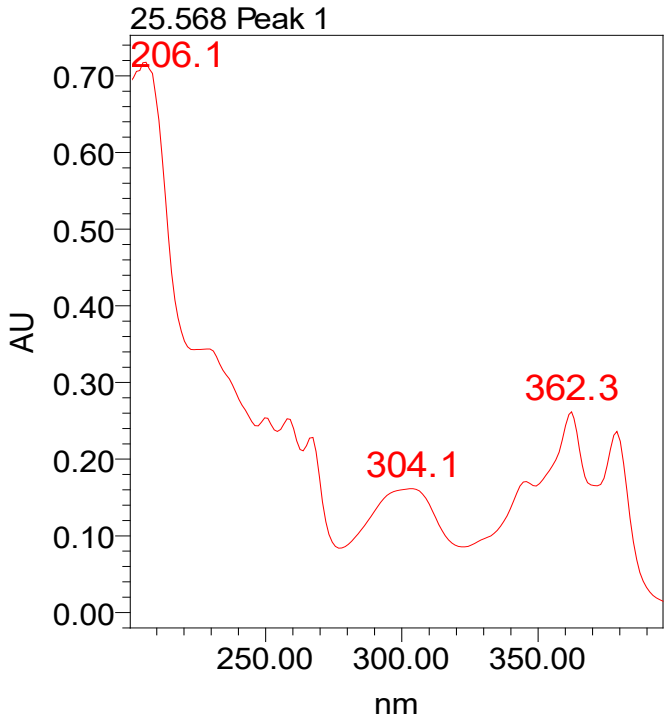

FIGURE 3. HPLC chromatogram and UV spectrum of canthin-6-one

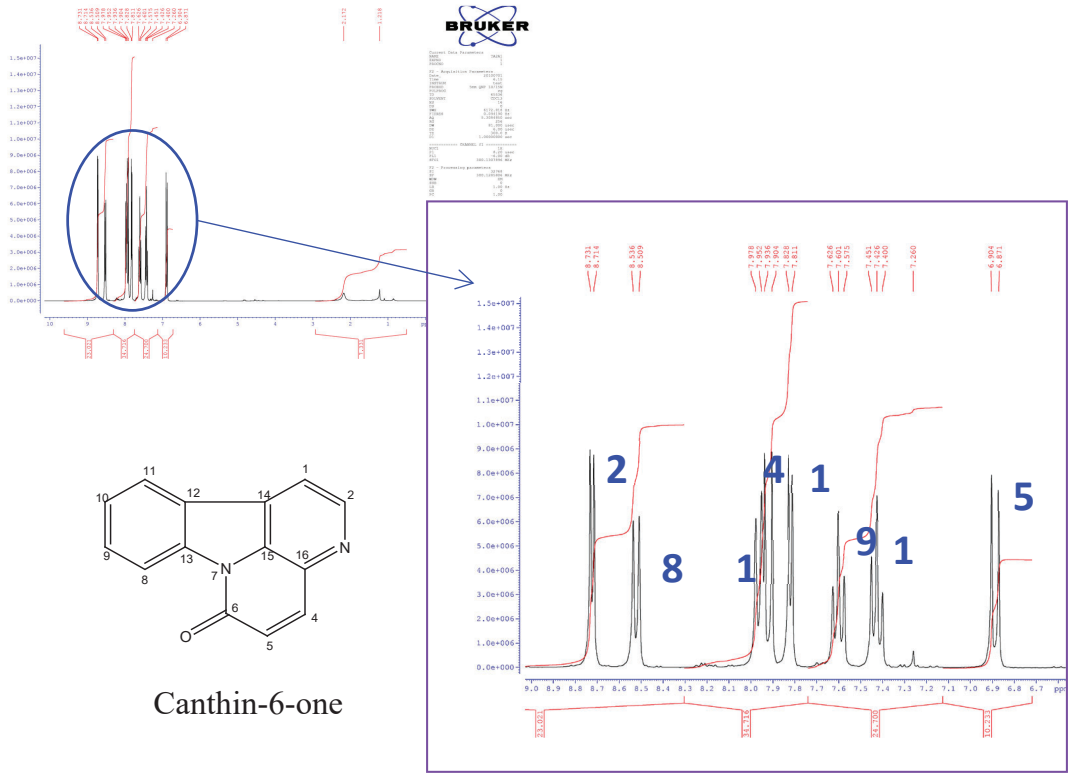

FIGURE 4. (a) Canthin-6-one

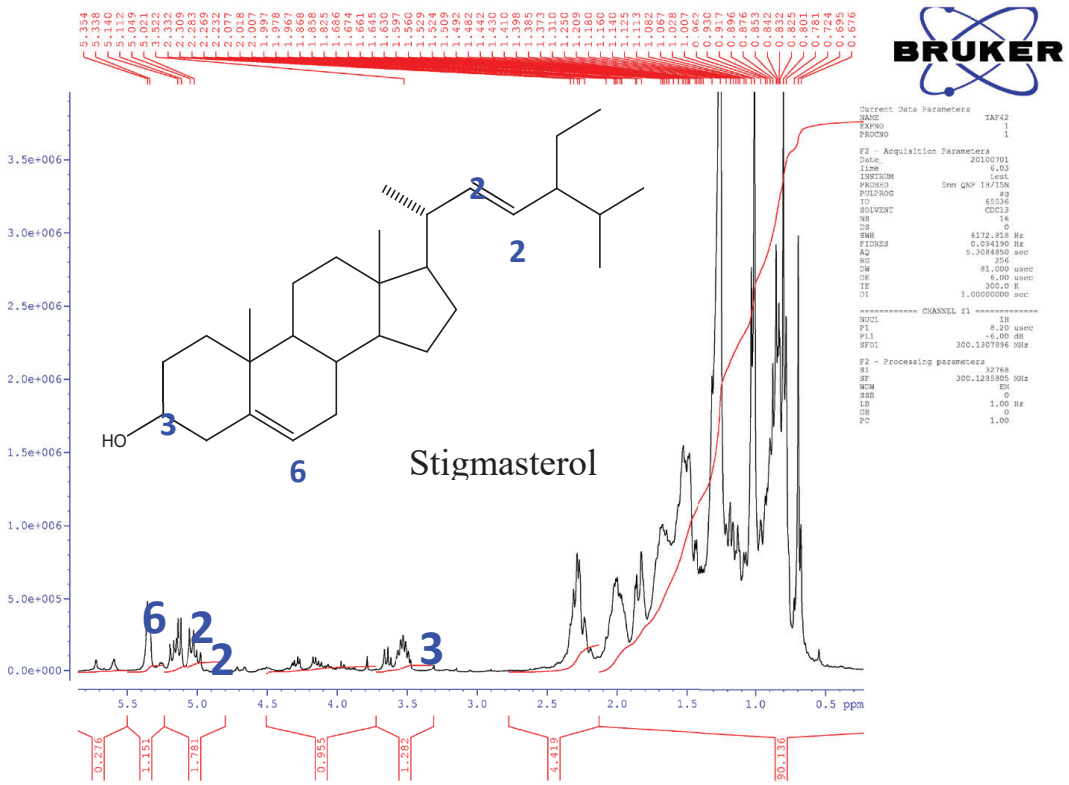

FIGURE 4. (b) Stigmasterol

the other two major compounds that have been detected in *E. longifolia* hairy roots culture are canthin-6-one and stigmasterol (Figure 4). The identification of these two chemical compounds were on the basis of proton and carbon NMR spectroscopic data and comparison with literature (Mitsunaga et al. 1994).

The production of canthin-6-one is also detected in *E. longifolia* roots and tissue culture plantlets as reported by Chua et al. (2011) and Hassan et al. (2012) and two novel canthin-6-one alkaloids, 4,9-dimethoxy-5-hydroxycanthin-6-one and 9-methoxy-(R/S)-5-(1-hydroxyethyl)-canthin-6-one, along with fifteen previously identified alkaloids, were isolated from the roots of *Eurycoma longifolia* Jack from Thailand by Zhang et al. (2020).

Several of *E. longifolia* pharmacological properties are attributed to quassinoids and canthin-6-one alkaloids. Canthin-6-one is an indole alkaloid found in the Simaroubaceae family alongside quassinoids and b-carboline alkaloids (Chaingam et al. 2021). Canthin-6-one was also found to have strong cytotoxicity to human lung cancer (A-549) (Kuo et al. 2004). Meanwhile, Choonong et al. (2022) reported that canthin-6-one alkaloids, including canthin-6-one-9-O-β-D-glucopyranoside, 9-methoxycanthin-6-one, canthin-6-one, and 9-hydroxycanthin-6-one, demonstrated PDE-5 enzymatic inhibition with IC_{50} values of 2.86±0.23, 3.30±1.03, 4.31±0.52, and 4.66±1.13 μM, respectively. The ethanolic extracts from the intact roots of *E. longifolia* and *E. harmandiana*, as well as the *in vitro* root culture of *E. harmandiana*, contained substantial amounts of canthin-6-one alkaloids (1.50±0.04, 2.12±0.03, and 3.48±0.08 mg/g dry weight, respectively), and exhibited strong PDE-5 inhibitory activity.

Aside from geographic location, metabolite concentration in plant samples is also affected by processing temperature. The constancy of the phytochemical composition is critical for the standardization. Choo and Chan (2002) developed a reversed Phase-High Performance Liquid Chromatography (HPLC) method with a photodiode array detector to determine three major alkaloids: 9-methoxycanthin-6-one, 3-methylcanthin-5,6-dione, and its 9-methoxy analogue in *Eurycoma longifolia* obtained from different sources.

Meanwhile, stigmasterol is also one of the bioactive compounds that has been isolated from *E. longifolia* plants as reported by Rehman, Choe and Yoo (2016). Stigmasterol is a plant sterol that can attach to chondrocyte membranes and has anti-inflammatory and anti-catabolic effects (Gabay et al. 2010).

Nazirah et al. (2018) and Tran et al. (2018) found that elicitation with jasmonic acid, yeast extract, methyl jasmonate, and salicylic acid can enhance the production of 9-methoxycanthin-6-one. Elicitation techniques have positive effects on the synthesis of chemical compounds and can be employed in the further study to boost the production of target compounds.

Raw materials for the phytochemical industry are largely sourced from natural forests. Exploitation can lead to the extinction of a plant species. Plucking the leaves and pruning the stems may keep a plant alive, but possessing the roots necessitates pulling the root from the earth, which might kill a tree. The root of the *E. longifolia* plant is the most desired component because it contains many bioactive chemicals. The uncontrolled practice of uprooting roots from their habitats has the potential to deplete natural resources. Hairy roots culture of *E. longifolia* is an alternative source for the production of *E. longifolia* chemical compounds for future used and further study.

CONCLUSION

Hairy root cultures of *E. longifolia* can produce canthin-6-one and stigmasterol, in addition to 9-methoxycanthin-6-one, which are chemical compounds of *E. longifolia*. To ensure the continuous production of these chemical compounds, hairy roots culture must be cultivated in a bioreactor and elicitation methods can be used in future studies to enhance the production of target compounds.

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