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Distribution of 9-methoxycanthin-6-one from the intact plant parts and callus cultures of *Eurycoma longifolia* (Tongkat Ali)

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Abstract

Study was carried out to determine 9-methoxycanthin-6-one distribution in intact plants and callus cultures of *Eurycoma longifolia.* Qualitative analysis using TLC revealed that the compound 9-methoxycanthin-6-one was present in leaves, petioles, stem, rachis, tap roots, fibrous roots, cotyledons and embryo of the *in vivo* plants. The quantitative analysis using HPLC showed that the highest concentration of 9-methoxycanthin-6-one content was found in tap roots (4.10 mg.g^{-1} dry weight (DW) followed by fibrous roots (3.91 mg.g^{-1} DW), rachis (2.10 mg.g^{-1} DW), cotyledons (1.44 mg.g^{-1}DW) and embryo (0.84 mg.g^{-1} DW). Petioles stem and leaves had relatively low concentrations compared to other intact plant parts, with 0.15 mg.g^{-1} DW, 0.12 mg.g^{-1} DW and 0.08 mg.g^{-1} DW, respectively. Compound 9-methoxycanthin-6-one was also present in callus tissues derived from different explants. The highest concentration was detected in fibrous root-derived callus (7.12 mg.g^{-1} DW tissues), followed by stem-derived callus (4.18 mg.g^{-1} DW tissues), leaf-derived callus (2.17 mg.g^{-1} DW tissues), embryo-derived callus (2.03 mg.g^{-1} DW tissues), rachis-derived callus (1.25 mg.g^{-1} DW tissues), tap root-derived callus (0.96 mg.g^{-1} DW tissues), petiole-derived callus (0.61 mg.g^{-1} DW tissues) and cotyledon-derived callus (0.18 mg.g^{-1} DW tissues). From the comparison between the data of using callus tissues and intact plant parts, it has shown that the higher concentration of 9-methoxycanthin-6-one of more than 73.7 % was detected in callus tissues.

Keywords *Eurycoma longifolia* . Callus. 9-methoxycanthin-6-one.

Abbreviations: DW_Dry weight; HPLC_High Performance Liquid Chromatography; TLC_Thin Layer Chromatography

Introduction

It is well known that plants produce a variety of economically important secondary metabolites approximately 4000 new discoveries are being made every year, and over 100,000 compounds are known (We et al., 2002). Some of the plant biochemicals are used in the healthcare, food, flavour and cosmetics industries (Frank and Masanaru 1995). Meanwhile, others are used for the production of agrochemicals, fragrances, colours and biopesticides (Pascal and John 2002). Examples of plant secondary metabolites used for the production of pharmaceuticals are dopamine, morphine, codeine, reserpine, and the anticancer drugs such as vincristine, vinblastine and taxol (Frank and Masanaru,1995). The plant material used in this study is *Eurycoma longifolia* Jack, which is also known locally as 'Tongkat Ali' in Malaysia and 'Pasakbumi', in Indonesia. *E. longifolia* is commonly used in traditional medicine against a variety of diseases (Ang et al., 1995; Chan et al., 1995). The roots are used in traditional medicine to increase male virility, sexual prowess and have gained notoriety as a male aphrodisiac (Kuo et al., 2003). Pharmacological evaluations on the various compounds isolated from *E. longifolia* showed that it also possessed anti-malaria (Kardono et al. 1991), anti-ulcer (Tada et al. 1991), cytotoxic (Morita et al., 1990; Kardono et al., 1991; Itokawa et al., 1992; Morita et al., 1993), antimalarial (Ang et al. 1995) and antipyretic properties (Chan et al., 1995). Kuo et al. (2003) reported that 9-methoxycanthin-6-one compound from the *in vivo* roots of this plant has demonstrated cytotoxicity against human lung cancer (A-549) and human breast cancer (MCF-7) cell lines. In addition, the high concentrations present in *E. longifolia* is might be due to several compounds including a variety of quassinoids (Jiwajinda et al., 2002). Previously, we have reported the factors affecting the accumulation of 9-methoxycanthin-6-one in callus cultures of this plant (Rosli et al., 2009). The present study was carried out to investigate the distribution and quantification of 9-methoxycanthin-6-one from the *in vivo* plant parts and callus from the *in vitro* cultures of *E. longifolia*.

Results and discussion

Distribution of 9-methoxycanthin-6-one in intact plant parts and callus cultures of E. longifolia using TLC and HPLC .

Compound 9-methoxycanthin-6-one from the roots of *E. longifolia* was reported to possess antitumor properties (Kardono et al., 1991). In this study, thin layer chromatography was used for separation and identification of 9-methoxycanthin-6-one in different explants of intact plant parts and callus cultures of *E. longifolia* using solvent mixtures of methanol and chloroform. Figure 1 shows the TLC separation of standard 9-methoxycanthin-6-one and eight samples of *E. longifolia* crude extracts from intact plant parts. The colour observed for standard 9-methoxycanthin-6-one under UV light was yellowish green. From the results, we have obtained the R_f value for the alkaloid which was 0.83. It was found that 9-methoxycanthin-6-one was detected in all different types of explants. The spot size in the chromatogram, showed the concentrations or the quantity of 9-methoxycanthin-6-one detected in the sample, which was significantly higher in tap roots than fibrous roots. Meanwhile, TLC separate and identify 9-methoxycanthin-6-one in callus cultures induced from different types of explants from *E. longifolia* (Fig. 2). It was found that 9-methoxycanthin-6-one was detected in all callus tissues derived from different explants. Based on the size of the TLC spots obtained in the chromatogram, the tap roots and the fibrous roots derived calluses produced the highest amount of 9-methoxycanthin-6-one. The results showed that large quantity of 9-methoxycanthin-6-one was present in calluses similar to intact plant parts. Further studies were carried out using HPLC analysis. The retention time of 9-methoxycanthin-6-one was 11.40 min. The results of HPLC analysis for different types of intact plant parts were shown in Fig. 3, Tap roots had the highest concentration of 9-methoxycanthin-6-one content (4.10 $mg.g^{-1}$) followed by fibrous roots (3.91 $mg.g^{-1}$), rachis (2.10 $mg.g^{-1}$), cotyledons (1.44 $mg.g^{-1}$), embryo (0.84 $mg.g^{-1}$), petioles (0.15 $mg.g^{-1}$), stem (0.12 $mg.g^{-1}$) and leaves (0.08 $mg.g^{-1}$). Meanwhile, the results of HPLC analysis for different types of callus cultures showed that the highest concentration of 9-methoxycanthin-6-one was detected in fibrous root-derived callus (7.12 $mg.g^{-1}$), followed by stem-derived callus (4.18 $mg.g^{-1}$), leaf-derived callus (2.17 $mg.g^{-1}$), embryo-derived callus (2.03 $mg.g^{-1}$), rachis-derived callus (1.25 $mg.g^{-1}$), tap root-derived callus (0.96 $mg.g^{-1}$), petiole-derived callus (0.61 $mg.g^{-1}$) and cotyledon-derived callus (0.18 $mg.g^{-1}$)(Fig. 3). As a conclusion, fibrous root-derived callus of *E. longifolia* contained highest amount of 9-methoxycanthin-6-one. It is relatively higher amount than the intact fibrous roots. The 9-methoxycanthin-6-one content was significant in all tested samples and higher concentrations were detected in roots than the other part of the plant. As a comparison, the concentration of 9-methoxycanthin-6-one detected was higher in callus tissues than in the intact plant parts. Roots have been demonstrated to play an important role for biosynthesis of plant secondary metabolites. Studies have shown that the roots were primarily responsible for biosynthesis of terpenoids, steroids, alkaloids and phenolic compounds in higher plants (Harborne 1991). Previously, it was reported that the roots were the main site for the biosynthesis of tropane alkaloids (Hashimoto et al., 1991). These alkaloids were then transported naturally through a vascular system (Kitamura et al., 1993) and accumulated in leaves or other parts of plants (Mano et al., 1989). In addition, Kardono (Kardono et al., 1991) stated that 9-methoxycanthin-6-one alkaloid compound could be obtained from the roots of *E. longifolia*.

Consequently from the above experiment, it was found that fibrous roots derived callus of *E. longifolia* was the best source to obtain the highest concentration of 9-methoxycanthin-6-one. As a comparison, the concentration of 9-methoxycanthin-6-one was detected to be higher in callus tissues than in the intact part. This result will be used in subsequent experiments to enhance the production of 9-methoxycanthin-6-one. Previously, we have published that the combination of different basal media, carbon sources, phytohormone, pH and amino acids could be a potential approach for the enhancement of 9-methoxycanthin-6-one compound via *in vitro* technology (Rosli et al., 2009).

Materials and methods

Plant materials

E. longifolia plants were grown at the Laboratory of Natural Product Discovery, Institute of Bioscience, Universiti Putra Malaysia (UPM) Serdang Selangor, Malaysia.

Sterilization of explants

The explants collected from *E. longifolia* plants were washed under running tap water for 30 minutes and were carried out surface sterilization according to our previously published report (Rosli et al., 2009). The sterilized explants were then cut into small pieces and then transferred into vials (8.4 x 2.4 cm).

Callus Induction

Callus induced from various explants (leaf, petiole, rachis, stem, tap root, fibrous root, cotyledon and embryo). Explants were excised and transferred to fresh medium. Callus cultures were multiplied and maintained by subculturing onto MS medium (Murashige and Skoog 1962) using 2.0 $mg.L^{-1}$ of 2,4-D at three week intervals. Calluses obtained from three week old callus cultures were used for the study.

Analysis of 9-methoxycanthin-6-one

Compound 9-methoxycanthin-6-one standard was provided by Professor Chan Kit Lam, School of Pharmaceutical Science, Universiti Sains Malaysia (USM) and was prepared as 5.0 $\mu g.mL^{-1}$ methanolic solution. Its isolation and purification were performed following the method as previously described (Choo and Chan, 2002). Standard curve was obtained by preparing different dilutions of 9-methoxycanthin-6-one stock solution and then was filtered through a Waters Sep-Pak Classic Cartridge (Waters, USA) prior to HPLC analysis.

Extraction of 9-methoxycanthin-6-one from Callus and Intact Plant Parts

The callus tissues and intact plant parts were harvested and dried in an oven at 45 ^{o}C for 48 hours before being pulverised using mortar and pestle. The extraction procedure used for extracting 9-methoxycanthin-6-one was as reported by (Choo et al., 2001) with minor modifications. One gram of dried

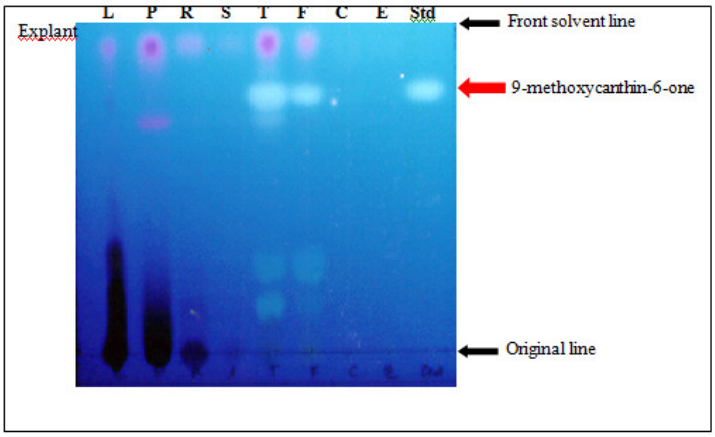

Fig 1. TLC spot of 9-methoxycanthin-6-one on aluminium sheets coated with Silica gel 60 F_{254} and developed with 25 DC-Alufolien Kiesel gel 60 F_{254} using chloroform and methanol solvent. L Leaf, P Petiole, R Rachis, S Stem, T Tap root, F Fibrous root, C Cotyledon, E Embryo, Std Standard 9-methoxycanthin-6-one.
R_f values 9-methoxycanthin-6-one = 0.83

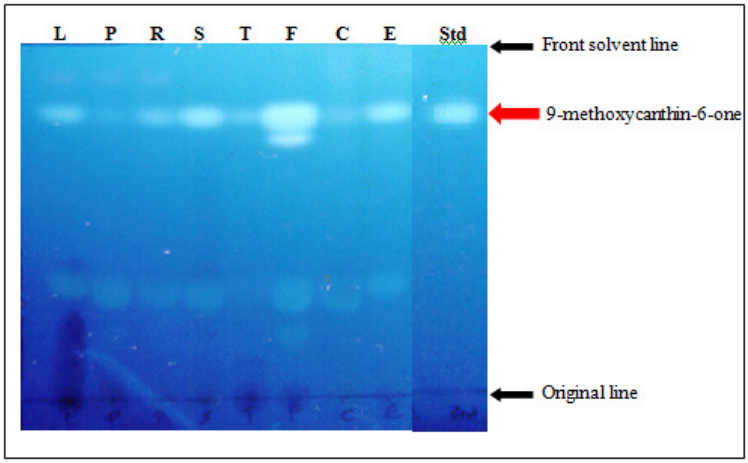

Fig 2. TLC spot of 9-methoxycanthin-6-one on aluminium sheets coated with Silica gel 60 F_{254} and developed with 25 DC-Alufolien Kiesel gel 60 F_{254} using chloroform and methanol solvent (Callus cultures). L Leaf, P Petiole, R Rachis, S Stem, T Tap root, F Fibrous root, C Cotyledon, E Embryo, Std Standard 9-methoxycanthin-6-one. R_f values 9-methoxycanthin-6-one = 0.83

samples was ground using pestle and mortar, before adding 20 mL of solvent 4:1 (CH_3OH_2: $CHCl_3$). The extract was then sonicated for 15 minutes. The homogenate was filtered through four layers of miracloth and centrifuged at 12 000 *g* for 10 min at 4°C. The supernatant was used for the thin layer chromatography (TLC) analysis. The extracts were further filtered through a Waters Sep-Pak Classic Cartridge (Waters, USA) for high-performance liquid chromatograph (HPLC) analysis. Quantitative determinations of the 9-methoxycanthin-6-one concentrations were carried out by the external standard method.

Analysis of 9-methoxycanthin-6-one using thin layer chromatography (TLC)

The analysis procedures for 9-methoxycanthin-6-one using (TLC) were modified from Choo et al. [16]. The samples were spotted on a TLC plate and were ran on the TLC plate with solvent (8 {$CHCl_3$,}: 2 {CH_3OH_2}). The TLC was performed on aluminium sheets coated with Silica gel 60 F_{254} (Merck catalogue no. 1.05554) and developed with 25 DC-Alufolien Kiesel gel 60 F_{254}. Compound 9-methoxycanthin-6-one was detected in fluorescence emission under irradiation of UV lamp (366 nm).

HPLC Analysis of 9-methoxycanthin-6-one

The analysis procedures for 9-methoxycanthin-6-one using HPLC were modified from Yam and Chan (2001). The crude extract of Tongkat Ali was analyzed with an Agilent 1100 Series HPLC (Agilent, USA) comprising Agilent Chem Station for LC System, a manual injector with external 20 µL sample loop, a diode array detector, a quaternary pump and vacuum degasser. Reversed-phase separations were carried out using a 3.9 x 150 mm I.D. Nova Pak C18 60A steel cartridge column; fitted at 4 µm (Waters Associates, USA, Part no. 36975) containing dimethyloctadecylsilyl boundedamorphous silica and methanol (CAS no. 67-56) was used at a flow rate of 1.0 $mL.min^{-1}$ at room temperature. The mobile phase consisted of a mixture of acetonitrile and distilled-deionised water (Elga, England) (pH 2.5) acidified with trifluoroacetic acid (TFA)(Sigma, USA) to pH 2.5

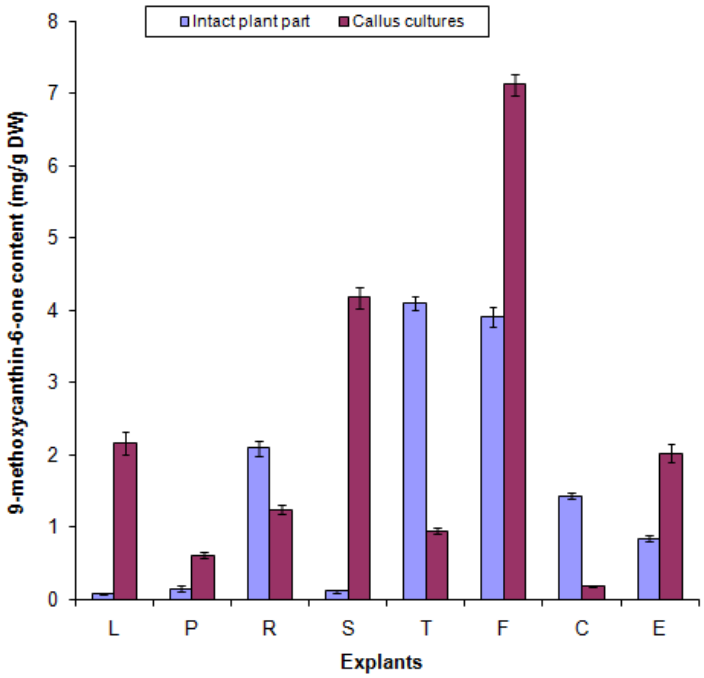

Fig 3. Distribution of 9-methoxycanthin-6-one in different plant parts and callus cultures of *Eurycoma longifolia.* L Leaves, P Petioles, R Rachis, S Stem, T Tap roots, F Fibrous roots, C Cotyledons, E Embryo, Std Standard 9-methoxycanthin-6-one. Bars indicate the standard deviation of 9-methoxycanthin-6-one determinations are from three replicates.

(40:60). The total running time was 30 min and the detection of 9-methoxycanthin-6-one compounds was monitored at the wavelength of 272 nm. The reference of 9-methoxycanthin-6-one was dissolved in HPLC grade methanol. The concentration of identified compounds was determined by external standard method.

Statistical analysis

The data were compared by one-way ANOVA following by a Tukey to compare means of the sample.

Conclusion

In conclusion, the experiment was carried out on the study of the 9-methoxycanthin-6-one distribution and its content in intact plant parts and callus tissues obtained from different explants types using TLC and HPLC. Semi-quantitative analysis using TLC revealed that 9-methoxycanthin-6-one was present in leaves, petioles, stem, rachis, tap roots, fibrous roots, cotyledons and intact plant embryo. By using HPLC, results showed that the highest concentration of 9-methoxycanthin-6-one was detected in tap root of the intact plant (4.10 $mg.g^{-1}$ DW). In addition, 9-methoxycanthin-6-one was also present in callus tissues derived from different explants. The highest concentration was detected in fibrous root-derived callus (7.12 $mg.g^{-1}$ DW) compared to other explants derived callus types. Experiment indicated that the concentration of 9-methoxycanthin-6-one was higher in callus tissues compared to the intact plant parts. Successful production of this important alkaloid in Tongkat Ali plant could be materialized and commercialized in a large-scale production by using bioreactor and hairy roots technology.

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