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Effect of Cell Source and pH of Culture Medium on the Production of Canthin-6-one Alkaloids from the Cell Cultures of Tongkat Ali (*Eurycoma longifolia* Jack)

Luthfi Aziz Mahmud Siregar[1], Chan Lai-Keng[1*], Boey Peng-Lim[2]

[1]School of Biological Sciences Universiti Sains Malaysia, 11800, Penang, Malaysia; [2]School of Chemical Sciences Universiti Sains Malaysia, 11800, Penang, Malaysia

Abstract

Callus and cell suspension cultures of *Eurycoma longifolia* Jack could be an alternative supply of 9-hydroxycanthin-6-one and 9-methoxycanthin-6-one. The callus tissues were initiated from leaves of different trees. The friable calli were used for the preparation of the cell suspension cultures of *E. longifolia*. The leaf explant of tree Eu-9 produced the most callus and also induced high cell biomass in the cell suspension culture, but it produced low quantity of 9-methoxycanthin- 6-one and 9-hydroxycanthin-6-one. The leaf explant from tree Eu-8 produced low quantity of callus and cell biomass, but produced the highest quantity of 9-methoxycanthin- 6-one and 9-hydroxycanthin-6-one. Optimum production of cell biomass was obtained on cell culture medium with pH 5.75 prior to autoclaving, but high alkaloids content could be induced in culture medium in acidic condition with pH 4.75 and 5.25 prior to autoclaving.

Key words: *Eurycoma longifolia*, Simaroubaceae, cell line, pH, 9-methoxycanthin-6-one, 9-hydroxycanthin-6-one

Introduction

Plants had been proven to contain various compounds that are used as dyes, pharmaceuticals, perfumes and insecticides. Plant cell cultures were also found to produce compounds characteristic of the original plant and this method was proposed as an alternative supply of these useful compounds (Hara et al. 1988; Ketchum et al. 1995; Verpoorte et al. 1993).

Eurycoma longifolia Jack, commonly known as Tongkat Ali in Malaysia, has been used as preparations of traditional medicines for decades (Nooteboom 1972). It belongs to the Simaroubaceae family and is widely found in the tropical forest in Malaysia, Indonesia, Thailand and Vietnam. The effectiveness of this plant as a form of medicine has been widely proven, using its extract and isolation of its active compounds. Quassinoids and canthin-6-one derivatives, tirrucallane-type triterpenes have been tested for antimalaria, antipyretic, anticancer, antiulcer, as well as aphrodisiac properties (Ang et al. 1997; Chan et al. 1986; Itokawa et al. 1992; Tada et al. 1991). The 9-methoxycanthin- 6-one and 9-hydroxycanthin-6-one has also been proven to possess cytotoxicity activities (Kardono et al. 1991).

The selection of growing cell line capable of producing biomass and active compounds was a crucial step. Study on cell lines selection and the effect of pH and MS macronutrients of the culture medium on biomass production in cell cultures of Tongkat Ali (*E. longifolia*) had been reported by us recently (Luthfi et al. 2003). In this paper, we investigated the effect of the different cell sources on the production of the two main alkaloids, 9-methoxycanthin-6-one and 9-hydroxycanthin -6-one. The cell biomass result reported in our previous paper (Luthfi et al. 2003) was used for this purpose and this will enable us to study the correlation between the cell biomass production and the production of alkaloids from each different cell line of *E. longifolia*. The effect of pH of *E. longifolia* cell suspension culture medium towards

* Corresponding author, E-mail: lkchan@usm.my
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production of the alkaloids was also investigated.

Materials and Methods

Determination of alkaloids content in the Callus Culture of *Eurycoma longifolia*

Callus tissues that were initiated from the previous experiment as reported in Luthfi et al. (2003) were used for determination of the two alkaloids (9-methoxycanthin-6-one and 9-hydroxycanthin-6-one) content. The callus tissues were produced from the leaves of nine different trees, named as Eu-1, Eu-2, Eu-4, Eu-5, Eu-6, Eu-7, Eu-8, Eu-9 and Eu-12, using Murashige and Skoog medium (MS) (1962) supplemented with 30 g/L sucrose and 10 mg/L NAA (1-napthaleneacetic acid). The calli were subcultured every four weeks and maintained on the same fresh medium. Ten samples were used for each line and the experiment was repeated two times. The alkaloids content was then determined for each callus source. The callus biomass data reported in Luthfi et al. (2003) was used to correlate between the production of biomass with the alkaloids content. Part of the induced callus from each source was used as material for the preparation of Tongkat Ali cell culture.

Determination of alkaloids content in the Cell culture of *Eurycoma longifolia*

The cells collected from the cell suspension culture, prepared previously in Luthfi et al. (2003), were used for the determination of 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one content. The cell suspension cultures were prepared by transferring one gram friable callus from each lines into 100 ml Erlenmeyer flask containing 20 mL MS liquid medium + X which was previously formulated (X was not revealed for possible patenting purpose). After three weeks, one gram of each cell line was transferred into the same liquid medium. Six samples were used for each cell line and the experiment was repeated two times. The cell cultures were placed on a gyratory shaker (G10 Gyrotory Shaker®, New Brunswick Scientific, N.J. U.S.A.) at 130 rpm at 25±2℃ with 24 h photoperiod of 1500 lux. Cell biomass was harvested and weighed after 14 days of culture and the biomass results reported in Luthfi et al. (2003). The biomass data was used to compare with the alkaloids contents determined for each cell line in this paper.

Effect of pH of culture medium on cell biomass and alkaloid production

Cell line Eu-9 of *E. longifolia* was found to be having the most rapid cell growth and hence was used in this experiment. One gram cell biomass was cultured in 100 mL Erlenmeyer flask containing 20 mL MS liquid medium + X with different pH. The pH of culture medium was adjusted with 0.1 M NaOH and 0.1 M HCl until the desired pH was obtained. Five different pH (4.75, 5.25, 5.75, 6.25 and 6.75) of the culture medium were used in this study. The cultures were maintained in the same culture conditions as mention above. Cell biomass was harvested and weighed after 14 days of culture and reported in Luthfi et al. (2003). The alkaloids content was determined from the harvested cells.

Extraction and analysis of alkaloids

The air-dried Tongkat Ali callus or cells (500 mg each) were extracted separately three times overnight by percolation in methanol at room temperature. The collected extracts were filtered and evaporated to dryness at 45℃ using a rotary evaporator. The dry residue was reconstituted in methanol and filtered through a Millipore (Whatman) filter (0.45 μm pore size) prior to high performance liquid chromatography (HPLC) analysis. The authentic standards, 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one, were obtained previously from Dr. Tripetch Kanchanapoom from Khon Kaen University, Khon Kaen, Thailand, and confirmed by comparison of its spectroscopic data with literature previously reported (Kardono et al. 1991; Kanchanapoom et al. 2002a; Kanchanapoom et al. 2002b). Determination of alkaloid was performed using a Shimadzu HPLC system Class *Vp* software program, with LC-10 AD*Vp* Shimadzu Liquid Chromatograph pump, a 20 μL injector loop connected to a reversed-phase 5 m Hypersil® ODS column 250x4.6 mm and SPD-10 A*Vp* Shimadzu UV-VIS detector. The mobile phase consisted of acetonitrile : 0.2% acetic acid (42:58) and the flow rate was 2.0 mL/min^{-1}. Alkaloid elution was routinely monitored at 280 nm and the alkaloids were identified on the basis of retention time. Quantitative determinations of the alkaloids concentration were calculated from calibration curve, putting in relation the relevant peak area with standard concentration. Eight standard solutions of the alkaloid (0.75 mg/L^{-1} 500 mg/L^{-1}) were analyzed for the calibration curve in triplicates. A separate curve was plotted on each day of analysis and its linearity was determined by regression line analysis. The results were presented as percentage of dried weight ± standard deviations.

Results and Discussion

High performance liquid chromatography (HPLC) and a UV detector at 280 nm were used for the analysis of alkaloids 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one contents

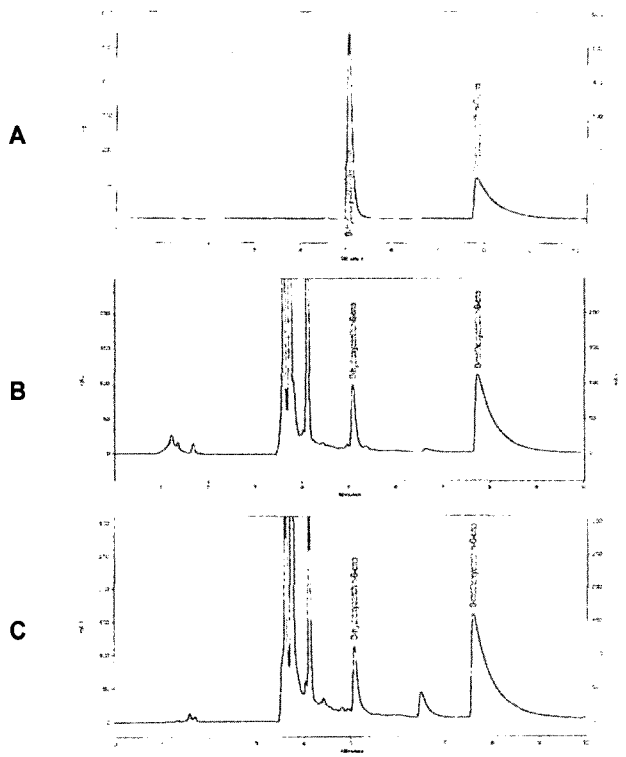

Figure 1. HPLC elution profile of the 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one of (A) standard solution, (B) *Eurycoma longifolia* callus Eu8, and (C) *Eurycoma longifolia* cells in MS liquid medium + X at pH 5.75.

from the callus and cell biomass of *E. longifolia* collected in the previous experiment (Luthfi et al. 2003). Alkaloids, 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one, were found to be present in the callus (Figure 1B) and cell culture (Figure 1C) of *E. longifolia*. They were confirmed as 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one with a retention time of 7.8 minutes and 5.1 minutes, respectively and found to have the same chromatogram pattern as that of the standard solution (Figure 1A).

The amount of calli initiated from different trees with different growth characteristics was found to be significantly different. The dried weight of calli correlated with their fresh weight. Leaf explants from Eu-8, Eu-9 and Eu-12 trees induced the most callus tissues as compared to other plant sources (Figure 2A). However, leaf explant from tree No. Eu-9 produced low quantity of alkaloid, only 0.001% of 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one were produced, as compared to other plant source. Leaf explant from tree Eu8, with high growth characteristic produced high alkaloids contents of 9-methoxycanthin-6-one (0.219%) and 9-hydroxycanthin-6-one (0.053%) as compared to other plants (Figure 2B). Leaf explant from tree Eu-7, with slow callus growth, produced higher alkaloid contents of 9-methoxycanthin-6-one and 9-hydroxycanthin-6-one than callus derived from trees Eu-9 and Eu-2 plant that had also fast growing characteristics. This indicated that the growth rate of callus tissues was not correlated with the alkaloids content. This strongly support the belief that the material source is an important factor in affecting the production of secondary metabolites in plant tissues as in *Catharanthus roseus* (Ganapathi and Kari 1990).

Result also showed that the leaf explant that induced fast callus growth not necessary produced more cell biomass, except leaf explant of tree Eu-9, which produced the maximum callus tissues and also induced high cell biomass in the cell suspension culture. Leaf explant from tree Eu-8 induced high amount of callus but after it was used for the preparation of cell suspension culture, only 3.137 g of cell biomass was produced (Figure 3A). This finding was also reported in our previous paper (Luthfi et al. 2003) and was applied here again for correlating with

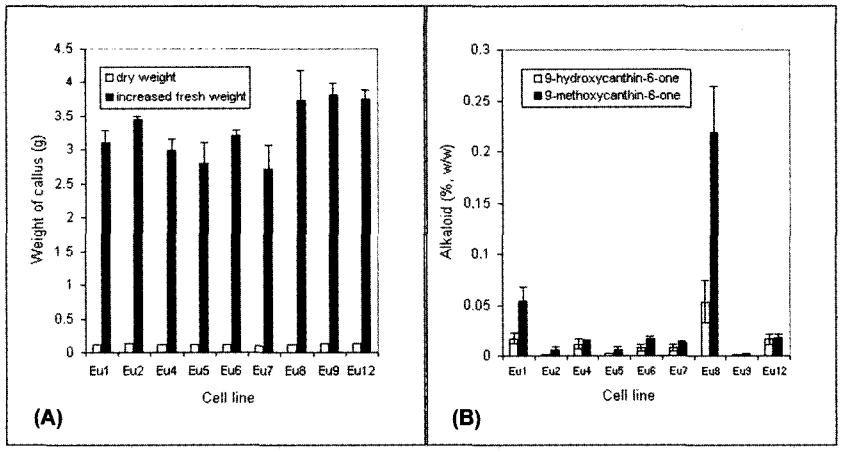

Figure 2. Production of callus (A) and content of alkaloid (B) from different leaves explant of *Eurycoma longifolia* in solid MS medium + 10 mg/L NAA + 30 g/L sucrose after 4 weeks of culture.

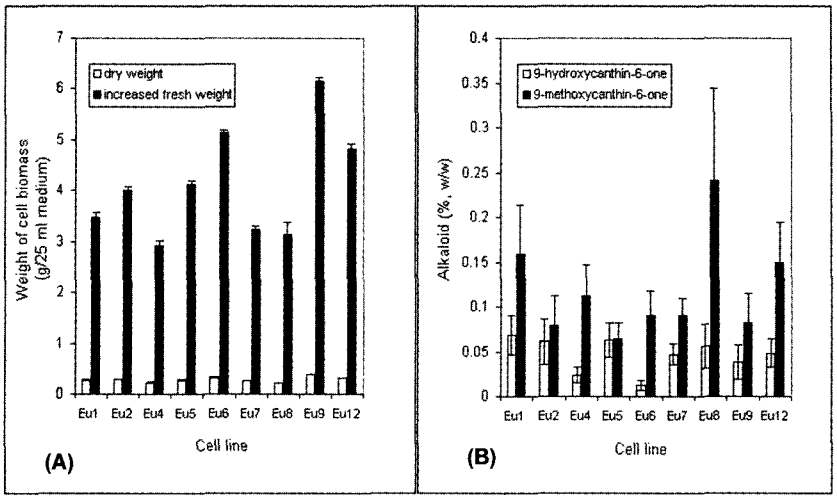

Figure 3. Effect of callus source on production of cell biomass (A) and alkaloid content (B) of *Eurycoma longifolia* cells in liquid MS medium + X after 14 days of culture.

their alkaloids content. Cell line Eu-8 was found to produce browning suspension culture before 12 days of culture, therefore, posting difficulty in subculturing and maintenance of this cell line. This clearly showed that fast growing callus not necessary produced more cell biomass. On the other hand, cell line Eu-8 which produced low cell biomass produced the highest quantity of alkaloid 9-methoxycanthin-6-one (0.241%) and relatively high 9-hydroxycanthin-6-one (0.056%). Cell lines Eu-1, Eu-2 and Eu-5 produced high quantity of 9-hydroxycanthin-6-one (0.069%, 0.061% and 0.063%, respectively) as compared to the other cell lines. Cell line Eu-9 with high growth characteristic produced only 0.080% of 9-methoxycanthin-6-one and 0.038% of 9-hydroxycanthin-6 -one (Figure 3B). The root extracts of the mature mother plant was found to contain lower content of 9-methoxycanthin-6-one (0.028 ±0.009%) and 9-hydroxycanthin-6-one (0.005±0.002%). This indicated that high yielding cell line coupled with the optimum culture medium could produce higher bioactive compounds from the plant cell cultures. In addition, the plant cells could be harvested within a short period as compared to their mother plants which took years to reach maturity. Tongkat Ali cells could be harvested after 14 days of culture. Several plant cell cultures such as *Coleus blumei* (Ulbrich et al. 1985), *Coptis japonica* (Fujita 1988), *Morinda citrifolia* (Zenk et al. 1985) and *Lithospermum erythrorhizon* (Fujita and Tabata 1987) also have been shown to produce large amounts of secondary metabolites as compared to their mother plants. However, the stability of the tissue and cell strains with respect to culture life is still a major concern in the industrial application of the cell culture process. Cell line screening could be achieved by comparative studies of different cell lines on the growth and bioactive compounds contents as had been done in this study and for ginseng, whereby the cell lines were selected based on the cell growth and saponin yields (Choi et al. 1994).

Culture media before cell inoculation that were too acidic or too alkaline could reduce the cell biomass production of *E. longifolia*. The best pH of the culture medium (MS + X) for optimum production of cell biomass was found to be 5.75 and the cell cultures in this medium were able to produce an increased cell wet weight of 5.035 g (dry weight of 0.309 g) within 14 days (Figure 4A). The dried weight of the cells corresponded with fresh cell biomass. High amount of 9-methoxycanthin-6-one was produced at acidic condition with culture medium at pH 4.75 (0.208%), while 9-hydroxycanthin-6-one was best produced at pH 6.25 (0.032%). Liquid culture medium at pH 5.75 prio to autoclaving also produced high cell biomass but producing only moderate amount of alkaloid (0.024% 9-hydroxycanthin-6-one and 0.149% 9-methoxycanthin-6-one). The amounts of both alkaloids produced were not significantly different when pH of the culture medium was 6.25 or 6.75 (Figure 4B). As reported by Payne et al. (1988), the concentration of hydrogen ions in the culture medium changed as the cells multiplied and produced secondary metabolites in the cell culture. Studies of *Catharanthus roseus* cell culture showed that uptake of indole alkaloids such as tabernanthine, ajmalicine and tryptamine could be increased by increasing the extracelular pH (Neumann et al. 1983). The pH shift between low and high values can also be used to release intracellular alkaloids into the culture medium (Payne et al. 1988).

To conclude, it was obvious that each *E. longifolia* cell line produced higher amount of secondary metabolites than its respective callus. Liquid culture medium (MS+ X) at pH 5.75 that stimulated high cell growth could also induce the production of both alkaloids, the 9-methoxycanthin-6-one and

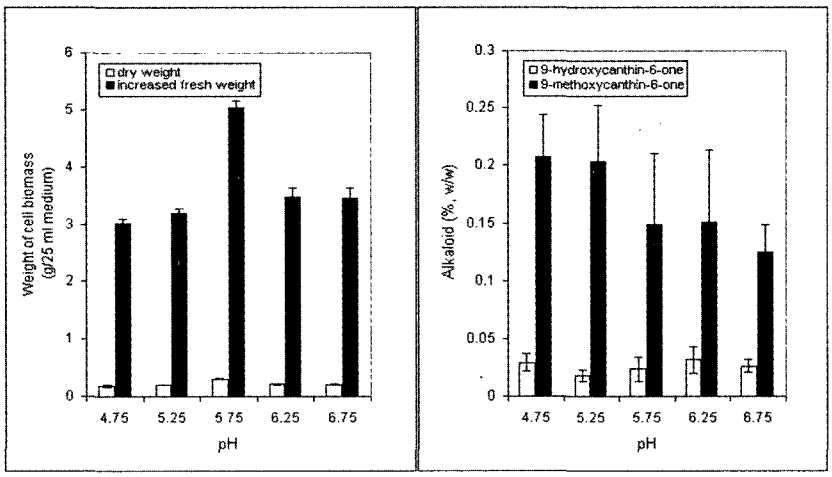

Figure 4. Effect of pH of culture medium MS + X on the cell biomass production and alkaloids content of *Eurycoma longifolia* cell suspension after 14 days of culture

9-hydroxycanthin-6-one from the cell cultures.

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