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Improved production of the indole alkaloid canthin-6-one from cell suspension culture of *Brucea javanica* (L.) Merr.

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Abstract: In an attempt to increase the productivity of the pharmaceutical compound canthin-6-one from cell suspension culture of *Brucea javanica*, a high cell density culture and improved culture conditions have been investigated with focus on culture conditions and stages of growth. The highest yield of canthin-6-one, 26.72 mg g^{-1} dry cell, was produced intracellular in 50 g L^{-1} cell mass. The established cell suspensions were harvested at the age of 40 days and placed into MS medium containing 1.0 mg L-1 2,4-D, 1.0 mg L-1 NAA, 0.1 mg L-1 Kinetin and 10 mg L^{-1} tryptophan for 33-40 days, approximately when the cultures were at their late stationary phase of growth. Addition of 20 mg L^{-1} tryptophan did not show a significant difference in canthin-6-one production ($p>0.05$). Higher than 10 mg L^{-1} of tryptophan showed adverse effect on the concentration of canthin-6-one alkaloid ($p=0.01$). Qualitative analysis of chloroform-extracts of dried cells from suspension cultures revealed spots on TLC; and the identity of canthin-6-one was established by comparison of its R_f value ($R_f=0.37$), its colour reaction with Dragendorff reagent and ammonium sulphate and its behaviour in UV with an authentic sample. Quantification of canthin-6-one alkaloid using TLC-densitometry scanner in response to different concentration of tryptophan as a precursor showed a concentration-dependent response. The production of canthin-6-one at 26.72 mg g^{-1} dried cell weight in this study, establishes a methodology of an improved procedure compared to those previously reported and offers an opportunity for production of canthin-6-one alkaloid at an industrial level.

Keywords: Canthin-6-One, cell suspension, *Brucea javanica,* secondary metabolites.

Introduction

The tree plant *Brucea javanica* (L.) Merr is a member of the family *Simaroubaceae,* which is known for its medicinal value to some Asian countries. The fruits of this plant have been used in the traditional medicine of Indonesia and China (Hasbi, 1979; Alam *et al*. 1995). In Indonesia the fruit is known as "Buah Makassar" and have demonstrated anti-malaria, anti-pyretic, and homeostatic effects. In China, the fruit is known as "Ya-Tan-Tze" and are used to treat malaria and amoebic dysentery and as an insecticide (Liu *et al*., 1990) and anti-cancer (Anderson *et al*., 1983).

The isolation of the indole alkaloid canthin-6-one from *Brucea* sp. has been reported by some workers (Harris *et al*., 1985; Liu *et al*., 1990). The compound and its hydroxylated and methoxylated derivatives have demonstrated cytotoxic effect and marked activities against malaria, leukaemia, carcinoma, keratinocytes of guinea-pig ear, and bacteria (Anderson *et al*., 1983).

Conventionally, indole alkaloid canthin-6-one is obtained from field grown intact plants. However, plants are seasonal and the quality of a metabolite may vary due to uncontrolled fluctuations in growing conditions and geographical variations. Growing intact plants is also confined to certain climate. In addition, because *Brucea* spp. are slow growing trees and the fruits are not readily available, cell culture offers an alternative method for the production of alkaloids. The expression of alkaloids in cell suspension is an interesting phenomenon as the alkaloids are not detectable in vegetative plant parts (Cordell, 1981).

The pattern of secondary metabolism in cell cultures is a combination of many interacting factors, including the physiological status of the culture, its origin (tissue type), age, and degree of development. Manipulation of the medium constituents and the physicals environments in which cell cultures are grown should be useful in improving the production of the characteristics secondary metabolites. Therefore, cell suspension cultures have several advantages; rapid growth rate, easy to manipulation, uniform, natural, offers the possibility of quality control and availability independent of climatic environment, and have limited number of cell types.

Canthin-6-one alkaloid has been expressed and successfully produced in cell suspension cultures of *Brucea* spp. The yield of this alkaloid was found to be two fold greater in the cells compared to that in the medium (Liu *et al*., 1990). While this suggests active excretion of alkaloids into the medium, the cells remain the target source

of alkaloid production. So far the yield of alkaloids from the cell suspension culture is considered low and, therefore, there is a need to further improve the procedure. Liu *et al*. (1990), have been successful in yielding 2 mg total alkaloids per 1 gram of cell dry weight. Nevertheless, they suggested further investigation of parameters necessary for maximizing yields of these compounds.

During the course of study an attempt has been made to maximise the yield of indole canthin-6-one using cell suspension culture and tryptophan precursor. So far, the use of tryptophan in stimulating alkaloid production from cell suspension culture of *B. javanica* has not been reported. However, Dicosmo and Towers (1984) have successfully used tryptophan to enhance indole alkaloid production in cell cultures of *Catharanius roseus*.

Materials and methods

Plant Material and Callus Culture

Fruits of *Brucea javanica* from Muntilan, Centre of Java province, were washed thoroughly and surface sterilised in 30 % Bayclin^{TM} (containing 5.5% sodium hypochlorite) for 15 minutes and then washed three times in distilled sterile water. Fruits were sliced and placed onto Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) containing 3% sucrose and 1.0 mg L^{-1} 2,4-D alone or with 1.0 mg L-1 NAA and 0.1 mg L-1 Kinetin at pH 5.8 for callus initiation and growth. Calli were routinely subcultured every four weeks. The latest passage of friable granulated and globular calli was used to initiate cell suspension culture for alkaloid production.

Establishment of Cell Suspension Culture

Cell suspension cultures were initiated by placing 5.0 g callus into 50 ml of liquid MS medium containing 1.0 mg L-1 2,4-D, 1.0 mg L-1 NAA and 0.1 mg L-1 Kinetin at pH 5.8 in 300 ml Erlenmeyer flask. The cultures were maintained at 25±2°C under illumination (photon flux density of approximately 150 μmol m^{-2} s^{-1}) on an orbital shaker with 2 cm stroke and subcultured every 10 days for four times. Treatments were applied by transferring 25 ml of cell biomass into liquid MS medium containing 1 mg L^{-1} NAA and 0.1 mg L^{-1} Kinetin and tryptophan precursor at 0, 10, 20, 40, and 80 mg L^{-1}.

Growth parameters of cell suspension

Cell suspensions used in the growth study were *preconditioned*, by inoculating 1:10 (w/v, 1.0 g cell pellet from the inoculum stock in 50 ml of liquid MS medium containing 1.0 mg L-1 2,4-D, 1.0 mg L-1 NAA and 0.1 mg L-1 Kinetin. Cultures were incubated on an orbital shaker at 120 rpm in darkness at 25 ± 2 C° for one week prior to use as inocula in various treatments.

Five replicates of 1.0 g of cells inoculated into 50ml of medium, supplemented with various concentrations of L-tryptophan, were incubated on an orbital shaker at 120 rpm in darkness at 25 ± 2 C° for a maximum of 40 days, and the cultures were assessed in eight time points; 0, 4, 12, 16, 21, 28, 33 and 40 day for the following characteristics (Arias-Castro *et al*., 1993; Torres, 1989):

Height of cell sediments (hcs) or settled cell volume was monitored during the incubation period using modified Erlenmeyer flasks with a branched measuring cylinder. *Packed cell weight (pcw)* was determined by centrifuging 25 ml of cell suspension cultures in a pre-weighed centrifugation tubes for one min at 1000 rpm, removing supernatant and weighing the packed cells. PCW is expressed as g L^{-1}. The height of cell sedimentation was monitored during the incubation period. *Dry cell weight (dcw)* was obtained by rinsing the pellets in 25 ml of distilled water and filtering the suspension on pre-weighed dry Whatman No.1 filter paper (Whatman Paper Ltd.) with suction, and drying cells at 60^0C for 48 h followed by weighing and expressed as mg ml^{-1}.

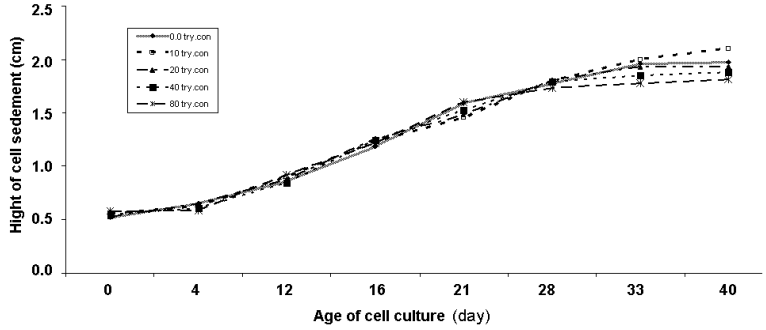
Analyses of cell culture with TLC for canthine-6-one alkaloids identification

Aliquots of 500 mg dried cell pellets of centrifuged (1000 rpm for 10 min) cell suspension cultures of *Brucea javanica* were ground in 25 ml of methanol and the extract was filtered and evaporated to dryness. The residue was extracted with 25 ml chloroform: water (1:1) mixture. The chloroform layer was separated and analyzed for canthin-6-one using TLC on silica gel (GF-254) eluted with toluene: acetyl acetate (6:4).

The aqueous extracts contained water soluble materials from samples of dried cells and authentic samples of canthine-6-one alkaloid, as a standard (generously donated by Professor Masayoshi Okano, University of Hiroshima, Japan), was brought to 85.5% with absolute ethanol. The suspension was vortexed for about 5 min and centrifuged at 3000g for 10 min. The supernatant fraction containing ethanol extractable materials samples along with the authentic samples of canthine-6-one alkaloid were directly used for TLC.

When the solvent has reached the top of the plate, the plate were removed from the solvent tray, air-dried, and the separated components of the mixture were visualized under UV lamp, fluoresced everywhere *except* where the organic compound were on the plate. Canthin-6-one compound was identified by direct comparison with authentic sample and quantified using

Fig.1. Height of cell sediments (cm) over time course of growth of Brucea javanica in cell suspension culture of 1.0 g pre-conditioned cells in 50 ml MS medium supplemented with NAA (1.0mgL-1), kinetin (0.1mgL-1) and different concentration of L-tryptophan.



densitometry. Spots were detected by UV, Dragendorff reagent and ceric ammonium sulphate, and quantified using densitometry at 355 nm with a TLC scanner (Shirnadzu CS-930).

Results and discussion

While production of alkaloids including canthin-6-one from cell suspension culture of *Brucea javanica* has frequently been reported by a number of workers (Harris *et al*., 1985; Liu *et al*., 1990), the highest total alkaloid obtained was only 2 mg g^{-1} dry cell weight (Liu *et al*., 1990). This low level of production is thought to be subject to improvement by stimulating the biosynthetic activity of cultured cells and optimization of culture conditions. In the industry of secondary metabolites, workers tend to patent the optimization of culture conditions found to increase the production to a commercial level of industrial value. The difficulty in accessing such information, therefore, emphasise the need for fundamental research to improve the expression of such compounds, of which canthin-6-one is of particular significance.

Induction of Embryogenic Callus

Enhanced production of embryogenic callus (EC) was achieved from the fruits of *Brucea javanica* on MS basal medium containing 1.0 mg L^{-1} 2,4-D, 1.0 mg L^{-1} kinetin and 3 % sucrose (85% EC) compared with MS basal medium with 1.0 mg L^{-1} 2,4-D alone (40% EC). The EC was characterised by a nodular, compact hard yellow-green cell clumps. Callus was maintained by selecting EC under a dissecting microscope and routinely subculturing callus lumps every four weeks onto the same medium for six passages.

Initiation of cell suspension and measuring growth parameters

Despite, the initiation of Embryogenic Cell suspensions (ECS) requires a relatively large amount of callus to serve as the an inoculum, in this study it was not feasible to initiate the cell suspension cultures by more than 1-2 g packed cell weight of EC clumps into 50 ml of liquid medium. Upon the addition of L-tryptophan precursor, the height of cell sedimentation (Fig. 1) increased gradually over the 40 days of incubation in all media with various concentrations. The effect of L-tryptophan on cell sedimentation height was not significant (p=0.01). However, there was a significant difference (p=0.01) in the cell sedimentation height due to the incubation period. The maximum sedimentation height was an average of 2.0 cm at 33rd and up to 40th days (Plate 1), indicating that cells reached its stationary growth point at the 40th day.

In addition, cells at the stationary phase of growth, while biologically active, invest most of the cell energy in the mitotic division for reproduction, once the cells approach the late stationary phase, this energy is believed to be redirected towards production of secondary metabolites. The stationary phase of growth was determined by monitoring cell sedimentation rate.

Usually, standard growth patterns, expressed as height of cell sediment, have an obvious initial lag period prior to any sign of cell division and rise in growth measures, which occur exponentially in a geometrical increase in the cell population. The cells then enter in a gradual deceleration in the division rate.

Finally, the cells enter a stationary or non-dividing stage. However, in Fig. 1, the growth curves of the cells of the three genotypes show

Plate 1. Eight-weeks-old embryogenic callus (EC) from the fruit of Brucea javanica (A) and its cell suspension (B) prepared from pre-conditioned cells MS medium supplemented with NAA (1.0mgL-1), kinetin (0.1mgL-1) and 10 mgL^{-1} L-tryptophan.

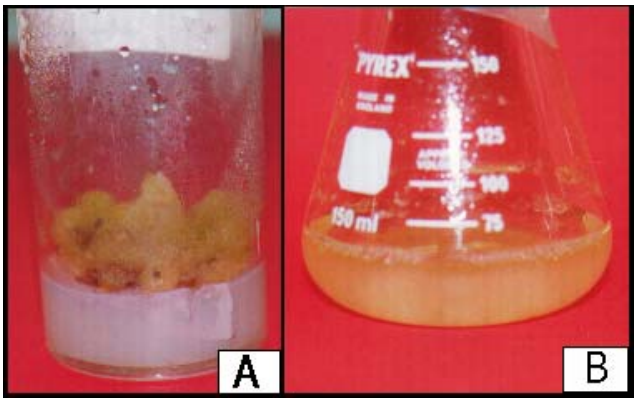


Fig.2. Comparison of mean Packed Cell Weight (PCW) of sediments of 40 days-old cells in suspension culture of pre-conditioned cells in MS medium supplemented with NAA (1.0mgL-1), kinetin (0.1mgL-1) and different concentration of L-tryptophan.

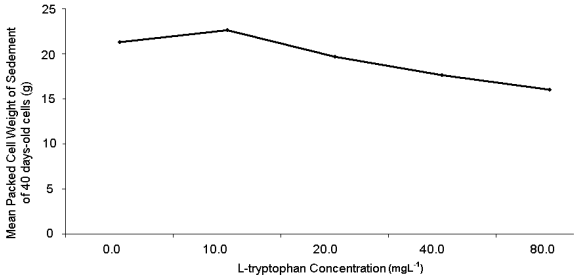
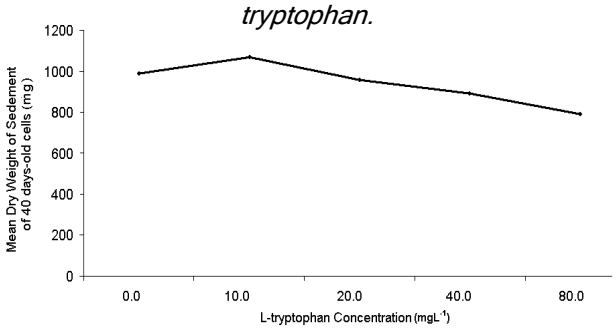


Fig. 3. Comparison of mean Dry Weight (DW) of sediments of 40 days-old cells suspension culture of pre-conditioned cells in MS medium supplemented with NAA (1.0mgL-1), kinetin (0.1mgL-1) and different concentration of L-tryptophan.



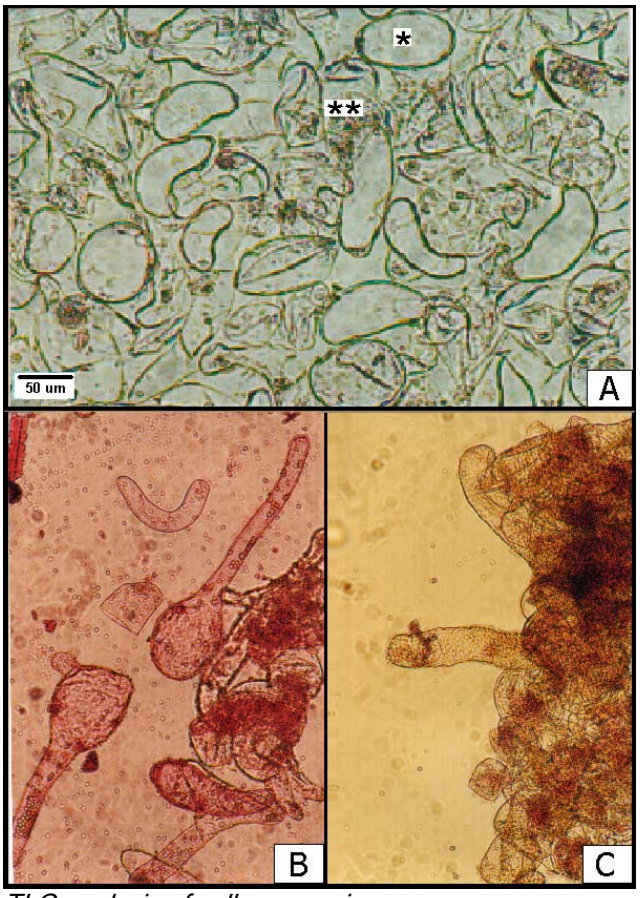
nearly S-shaped patterns with no substantial lag phase. This was due to the status of the inoculum, where cell suspensions were preconditioned so that the cells were actively dividing at inoculation. The growth measurements showed a rapid increase in packed cell weight and cell dry weight over the 40 days in culture, showing signs of decreasing rates.

On the contrary, the relationship between time of incubation and mean growth measurements of the ECS cultures including packed cell weight and cell dry weight is shown in Fig. 2 & Fig. 3. They show that cell packed cell weight (PCW) and dry weight (DW) at the 40th day of incubation varied significantly (p=0.01) due to the various concentration of L-tryptophan; showing highest values at a concentration of 10 mgL-1, above which the increasing concentration showed a retarded PCW and DW of cell biomass.

Upon microscopic examination, cell cultures in the MS medium supplemented with 1.0 mg L-1 2,4-D, 1.0 mg L-1 NAA and 0.1 mg L-1 Kinetin were composed of two types of cells. Most common was EC characterized as actively-dividing small cells with dense cytoplasm and prominent nucleus (Plate 2, A&B). The other type was *Non-embryogenic* cells (NEC) which were large, vacuolated and often elongated with sparse cytoplasm (Plate 2, C).

Since the metabolism of secondary products seems to be correlated with organized cell structures (Spencer *et al*., 1990), the embryogenic features of cells may reflect their totipotent nature and potential in expressing common secondary metabolites, especially canthine-6-one alkaloids.

Plate 2. Morphology of embryogenic cells (EC) and non-embryogenic cells (NEC) in cell suspension cultures of Brucea javanica. ***(A):*** *EC in 40-days-old cultures showing *cells with dense cytoplasm and prominent nucleus and **debris;* ***(B):*** *EC in 40-days-old cultures showing elongated cells with embryonal heads and* ***(C):*** *NEC large, vacuolated and aggregated cells with sparse cytoplasm.*



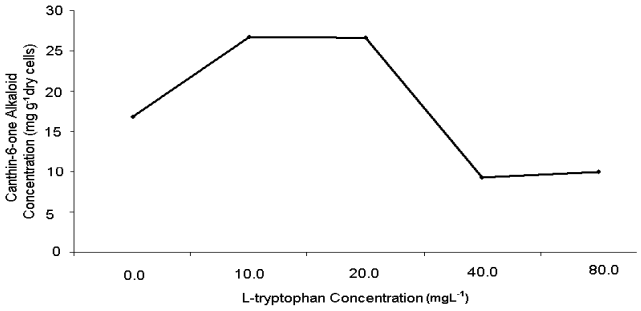
TLC analysis of cell suspensions

Compared to the spots of authentic canthine-6-one alkaloid standard at R_f value Rf = 0.37, tentative TLC-analysis detected no traces of canthine-6-one alkaloid in the supernatant fraction containing ethanol-extractable samples of cells.

However, high level of canthine-6-one alkaloid was detected in the extracts of the biomass of cell harvest. Quantitation of canthin-6-one alkaloid using TLC densitometry scanner in response to different concentration of tryptophan as a precursor showed a concentration-dependent response (Fig. 4) corresponding with the area under the curve (Fig. 5).

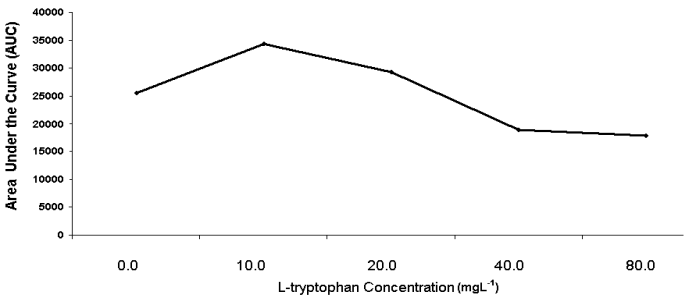
Addition of 10 mg or 20 mg L^{-1} of tryptophan maximized the production of canthin-6-one from

Fig. 4. Concentration of canthin-6-one alkaloid (mg g-1) in cell suspension culture of Brucea javanica in MS medium supplemented with NAA (1.0mgL-1), kinetin (0.1mgL-1) and different concentration of



cell suspension culture of *B. javanica*. On addition of 10 or 20 mg L^{-1} tryptophan, the concentration of canthin-6-one reached 26.72 mg g^{-1} dried cell weight but the two concentrations of tryptophan did not show a significant difference in canthin-6-one production (p=0.05). Higher or lower concentration of tryptophan showed adverse effect on the concentration of canthin-6-one alkaloid (p=0.01), as it might limit cell growth and have toxic affect on cells. It appears that, while tryptophan is proved to be an essential precursor for improved yield of canthin-6-one alkaloid, there is no linear relationship between the concentration of tryptophan and canthin-6-one accumulation.

Fig. 5. Area Under Curve (AUC) of canthin-6-one alkaloid in test cell samples in different L-tryptophan concentrations (mgL-1).



The stimulation of secondary metabolite production was found to occur with the addition of L-tryptophan (Dicosmo & Towers, 1984). Previous studies have had limited success in producing canthin-6-one alkaloid at a reasonable concentration from cell suspension cultures of medicinal plants. Liu *et al*. (1990) obtained only 2mg g^{-1} dry cell weight of total indole alkaloids from cell suspension culture of *B. javanica*. Anderson *et al*. (1983) obtained only 12.7 mg g^{-1} dry cell weight of canthin-6-one from cell suspension culture of *Alianthus altissima* (Osoba & Roberts, 1993). The production of canthin-6-one at 26.72 mg g^{-1} dried cell weight in this study, establishes a methodology of an improved procedure compared to those previously reported and offers an opportunity for production of canthin-6-one alkaloid at an industrial level.

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