

Effect of different media, sugars, illumination and temperatures on the production of styrylpyrone derivatives in callus culture of *Goniothalamus umbrosus*

(Kesan terhadap perbezaan medium, karbohidrat, cahaya dan suhu terhadap penghasilan terbitan stirilpiron di dalam kultur kalus *Goniothalamus umbrosus*)

A.K. Mohamad Kamal*, S. Subari*, H.L.P. Azimahtol**, R. Suri* and H. Aminuddin*

Key words: cell culture, callus, *Goniothalamus umbrosus*, styrylpyrone derivatives

Abstract

Cell cultures of *Goniothalamus umbrosus* represent an alternative to whole plant extraction as a source of styrylpyrone derivatives (SPD). The aim of this study was to optimize the cell growth and production of SPD in callus culture of *G. umbrosus*. Growth was measured by fresh and dry weights of the calli and the content of SPD produced was determined by HPLC. The callus cultures of *G. umbrosus* were established on MS medium supplemented with 3% sucrose with the combination of hormone, 2,4-D (3.0 mg/litre) and BAP (0.5 mg/litre). The cultures were incubated at 25 ± 2 °C and under white light condition. Maximum growth of callus culture was 31.75 ± 2.25 g fw/culture and 4.73 ± 0.18 g dw/culture at 6 weeks of incubation. The SPD accumulation in callus varied from 0.041–1.96 µg/g dw sample.

Introduction

In previous investigations, the genus *Goniothalamus* of Annonaceae family such as the bark of *G. giganteus* produced the secondary metabolite of the tetrahydrofuranopyrone, altholactone and goniothalenol. Jewers et al. (1972) also extracted secondary metabolite styrylpyrone derivative (SPD) from *Goniothalamus* sp. It was claimed that secondary metabolite from *Goniothalamus* sp. has many beneficial bioactive effects on human health, such as antitumor, antiaging, antistress and antimalaria.

Azimahtol and Johnson (1998) reported the extraction of styrylpyrone derivatives (SPD) from the stem, leaves and seeds of *G. umbrosus*, a plant endemic to East Malaysia. According to Azimahtol and Johnson (1998) *Goniothalamus* spp. is the only commercial source of SPD. Earlier study reported that SPD exhibits antifertility effect on early stage of pregnant mice (Azimahtol and Radhakrishnan 1994). Azimahtol and Johnson (1998), also reported that SPD is a potential antitumor agent in breast cancer. Secondary metabolite with antitumor activity plays an important role among drugs of natural origin. On the other

*Food Technology Research Centre, MARDI Headquarters, Serdang, P.O. Box 12301, 50774 Kuala Lumpur, Malaysia

**School of Bioscience and Biotechnology, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Selangor, Malaysia

Authors' full names: Mohamad Kamal Abdul Kadir, Subari Shibani, Azimahtol Hawariah Lope Pihie, Suri Roowi and Aminuddin Hussin

E-mail: mdkamal@mardi.my

©Malaysian Agricultural Research and Development Institute 2006

hand, interest is focused on the formation of such plant product by cell and tissue culture systems to manipulate and improve the product syntheses in those systems.

The study on the production of SPD from *G. umbrosus* tissue (callus) culture was first documented in 1998 (Imam 1998), although it was probably not widely known. Preliminary studies were using callus derived from leaf, seed, stem and root. The production of SPD from *G. umbrosus* callus is lower in yield compared to the mother plant (Imam 1998). The use of the plant cell culture process was looked at as a potential alternative for the more efficient production of active ingredients such as SPD. Strategies to increase the production of secondary metabolite should be considered. Manipulation of the plant cell culture environment and media can affect both the rates of growth and accumulation of secondary metabolite. The idea, of course is to identify a rapidly-growing cell line that can produce large amounts of secondary metabolite (Gibson et al. 1993). For example, the production of shikonin by *Lithospermum erythrorhizon* cultures is increased when the MS medium is changed to modified MS.

This method enables us to focus on producing multiple cell line and optimizing media for maximal cell growth and to achieve our goal of SPD production using plant cell culture.

Materials and methods

Cell lines and maintenance

Callus of *G. umbrosus* was established on MS medium as described previously by Imam (1998). Cultures were maintained on MS medium fortified with 3% sucrose, 3 mg/litre 2,4-D and 0.5 mg/litre BAP. The medium was solidified with 0.1% agar after adjusting the pH to 5.6–5.8 and was sterilized by autoclaving at 121 °C for 20 min. Amino acid, sugars and growth supplements were incorporated into the medium before autoclaving.

The incubation of callus line was maintained under the 16 hours light and temperature conditions at 25 ± 2 °C. The callus was subcultured at regular period of 30 days.

Effect of modified MS

Three medium formulations (modified MS) were studied. The formulations included MS major salt, MS with B5 vitamin (replacing MS vitamin) and half strength of MS medium (MS1/2) supplemented with 3% sucrose, 3 mg/litre 2,4-D and 0.5 mg/litre BAP.

Effect of carbohydrate (CHO)

The effect of CHO on callus culture was studied. The 3% concentration each of sucrose, glucose and fructose was supplemented in the MS medium with 2,4-D (3 mg/litre) and BAP (0.5 mg/litre).

Effect of light (illumination conditions)

Callus obtained was evaluated for different types of illumination conditions. The callus was incubated under the dark, bright white cool fluorescence light (36 watt lamp) (Philips, Malaysia) and 'agrolite' light (40 watt lamp) (Philips, Malaysia).

Effect of temperature

Callus obtained was evaluated for different temperature conditions. The callus was incubated at 15, 23, 25 and 31 °C in incubator (Tuntosec Tanaka).

Analytical procedures

Growth of callus was measured by fresh (fw) and dry weight (dw) samples. For dry weight measurement, the sample was dried in the oven at 50 °C for 24 h until the sample weight was stable. Values of fw and dw were recorded with mean of three replicates.

The SPD in callus cultures was extracted by solvent extraction. About 0.1 ± 0.02 g dw callus was extracted in 1.0 ml of ethanol and centrifuged at 10,000 rpm (10 min) for several times until the

extractant became colourless. The extractant was measured for its SPD production by using High Performance Liquid Chromatography (HPLC) at 240 nm (UV detector) using the following instrumental parameter: mobile phase: 45% v/v acetonitrile with pH 5.8; flow rate: 1.0 ml/min and column: 3.9 x 150 mm C18 reverse phase.

Results and discussion

Callus culture

Callus culture from seed explants *G. umbrosus* was developed according to Imam (1998). Cultures were maintained for



Plate 1. Callus derived from seed of *Goniiothalamus umbrosus* cultured on the basal MS medium supplemented with 3% of sucrose, 3.0 mg/litre 2,4-D and 0.5 mg/litre BAP under light condition after 5 weeks of incubation

more than one year by regular subculturing. The production of callus through subcultures was used for the growth pattern and to determine the SPD content. Callus induction was observed within two weeks after subculturing on MS medium containing different concentrations of 2,4-D, BAP, kinetin and picloram (0.1–5.0 mg/litre). The best callus induction was observed at 3.0 mg/litre (w/v) 2,4-D and 0.5 mg/litre (w/v) BAP with 3% of sucrose under light condition at 27 ± 2 °C (Plate 1). On this media composition, the callus produced was greenish white. The percentage of callus induction was 80–90 respectively. The same medium and conditions were used for the subculture.

Callus was established on basal MS medium supplemented with 3.0 mg/litre 2,4-D and 0.5 mg/litre BAP. The growth pattern of *G. umbrosus* was assessed by fresh and dry weight analysis by weeks from established media. Results indicated that the maximum callus growth was 31.75 ± 2.25 g fw/culture and 4.73 ± 0.18 g dw/culture after 6 weeks of incubation (Figure 1). A lag phase of about 2 weeks was observed, followed by rapid growth thereafter. Growth attained its maximum on the sixth week followed by slight decline, possibly due to

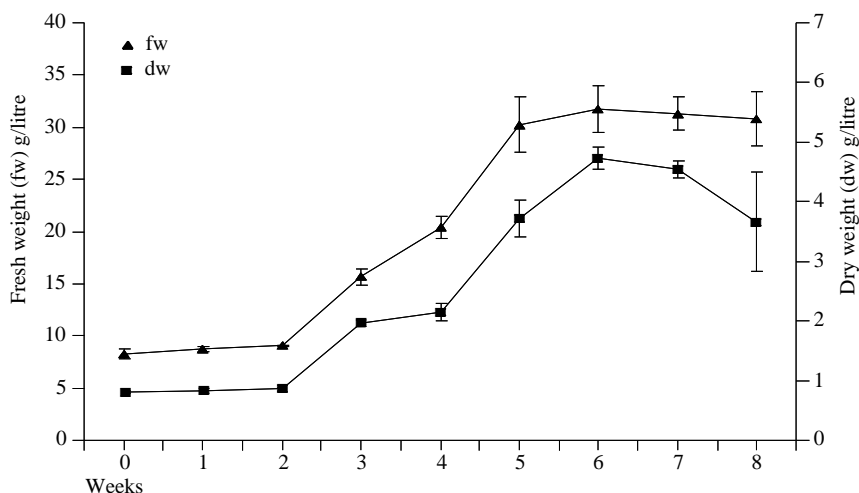


Figure 1. Time courses of fresh and dry weight of *Goniiothalamus umbrosus* callus culture incubated at 25 ± 2 °C under light condition. Bar indicates standard error of mean ($n = 3$)

deficiency of nutrient medium and cell death. Stable callus lines with subculture period of 5–6 weeks were maintained.

Production of SPD

The study on SPD production in mother plant and callus culture on solid medium in the production medium with 3% sucrose and 3.0 mg/litre (w/v) 2,4-D and 0.5 mg/litre (w/v) BAP showed that the SPD content was 300 times higher in mother plant (seed) ($346.12 \pm 12.52 \mu\text{g/g dw}$) compared to callus derived from seed explants ($0.041\text{--}1.099 \mu\text{g/g dw}$). This observation showed that cell culture technique on MS medium with 3% sucrose and 3.0 mg/litre (w/v) 2,4-D and 0.5 mg/litre (w/v) BAP was effective in promoting cell growth and SPD content. According to published report, other secondary metabolites such as taxol, anthraquinones and group of phenylpropanoids, alkaloids and terpenoids were detected in cell culture.

The SPD compounds in callus were analysed from the early stage to the death phase of growth. In callus, the highest production of SPD compounds was found during the death phase (slow-growing or non-growing culture). According to Rokem and Goldberg (1985), the production of plant secondary metabolites through cell culture produces higher concentration in slow-growing or non-growing culture.

Effect of medium formulation

The half strength MS medium (MS1/2) and the addition of higher concentration of thiamine (MSGB5) played essential roles in the growth and SPD content in the culture of *G. umbrosus*. Except for MS1/2, the other formulations tested were capable of supporting the cell growth. The culture growth was reduced to 50% when the concentration of MS medium was lower (about 50%). The maximum cell concentration on day 30 obtained in MS ($6.12 \pm 0.39 \text{ g/litre}$) and MSGB5 ($5.24 \pm 0.38 \text{ g/litre}$) medium was about 50% higher than MS1/2 ($2.66 \pm 0.17 \text{ g/litre}$) (Figure 2).

Previous study by Imam (1998) confirmed that the best medium for cell growth in culture tissue of *G. umbrosus* is the MS medium.

The production of SPD in basal MS medium and MSGB5 was higher than in MS1/2. The maximum SPD production on the early exponential phase in MS ($1.01 \pm 0.05 \mu\text{g/g}$) and MSGB5 ($0.912 \pm 0.02 \mu\text{g/g}$) was higher than MS1/2 ($0.41 \pm 0.02 \mu\text{g/g}$). According to Endress (1994), the effect of high concentration on the addition of thiamine in MSGB5 medium correlates with the increase on the secondary metabolite in plant tissue culture, but the medium did not influence any significant changes on the SPD production and cell growth (Figure 2). On the other hand, MS medium was effective in promoting both cell growth and SPD content. Hence in this study, MS medium was chosen as the medium to undergo further investigation.

Effect of sugar

Sucrose, glucose and fructose were tested for callus induction and SPD production. Each carbohydrate was added at the same molarity (3% of concentration). After the first month of regeneration, the yellow

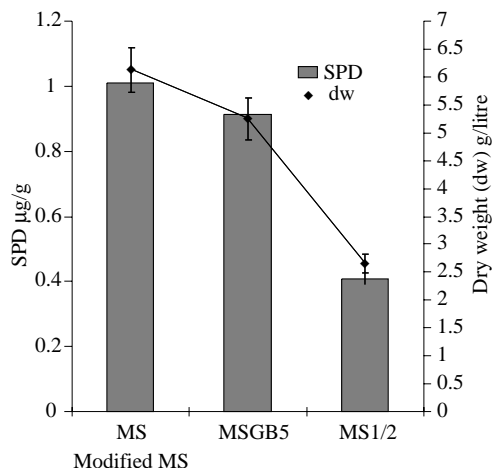


Figure 2. Effect of modified MS on growth (dw) and SPD production of *Goniothalamus umbrosus* callus cultures. Bar indicates standard error of mean ($n = 3$)

colour of callus on the medium containing sucrose turned greenish and yellowish. The yellow callus turned dark brown when the medium contained fructose and glucose. The maximum cell growth of culture was obtained on day 30 in medium treated with sucrose (7.53 ± 0.43 g/litre dw) compared to glucose (3.19 ± 0.13 g/litre dw) and fructose (2.69 ± 0.02 g/litre dw) (Table 1). Among the different carbon sources tested, sucrose was highly effective in the induction and development of callus. According to Marezki et al. (1974) sucrose is the best carbon source for the growth of most plant cell cultures.

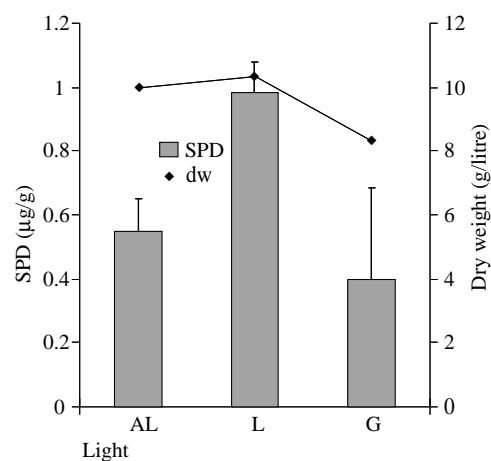
Sucrose gave the best medium for the SPD production (Table 1). Other sources of CHO such as glucose and fructose did not detect SPD in the callus culture. The results in Table 1 also indicated that the dw of callus was lower on medium containing glucose, while on fructose medium callus did not produce SPD. It was reported that sucrose was favourable to SPD accumulation in *G. umbrosus* plant cell cultures. According to Zenk et al. (1975) from 14 carbohydrates tested on *Morinda citrifolia* cell cultures, sucrose also gave the higher yield of antraquinone.

In nature, CHO is transported within the plant as sucrose and the tissue may have the inherent capacity for uptake, transport and utilization of sucrose. In the present study, carbohydrate such as fructose and glucose inhibited callus growth and SPD production completely. The inhibitory effect on culture may be due to lack of proper uptake and transport and subsequently utilization of the substances by the tissue

(Eapen and George 1990). Among the three types of sugars tested, only sucrose gave a significantly higher growth and SPD production than others.

Effect of illumination

Callus cultures on basal MS medium supplemented with 3% of sucrose and 3.0 mg/litre 2,4-D and 0.5 mg/litre BAP were treated with different conditions of light (bright white fluorescence light, dark and agrolite light conditions). Callus incubated under the dark, agrolite and light conditions were 8.33 ± 0.014 , 10.00 ± 0.017 and 10.33 ± 0.017 g/litre, respectively (Figure 3). Callus culture exposed to agrolite and light conditions showed very similar growth and did not



Note: Callus previously induced under light condition and subcultured under dark (G) and agrolite light (AL)

Figure 3. Effect of light on growth (dw) and production of SPD in *Goniothalamus umbrosus* callus cultures. Bar indicates standard error of mean ($n = 3$)

Table 1. Effect of the different carbohydrates on callus growth, colour, texture and SPD content after 30 days of culture ($n = 5$)

CHO	Colour	Texture	Dry weight	SPD content
Control	Dark brown	Soft	2.71 ± 0.02	nd
Sucrose	Greenish & yellowish	Hard & friable	7.53 ± 0.43	1.42 ± 0.04
Glucose	Dark brown	Friable	3.19 ± 0.13	nd
Fructose	Dark brown	Soft	2.69 ± 0.02	nd

nd = not detected

show any significant difference on growth rate. The maximum SPD content under light, agrolite and in the dark conditions was 0.98, 0.55 and 0.39 $\mu\text{g/g dw}$, respectively. The highest production of SPD was observed in pretreatment under light condition compared to agrolite and dark.

Callus grown under light and agrolite conditions were green in colour compared to those culture under dark condition (brownish) (data not shown). This suggested that chlorophyll might play an important role in the production of SPD. Schoofs et al. (1983) reported that accumulation of plant secondary metabolites in photoautotrophic cell cultures is based on the hypothesis that green callus would expedite the biosynthesis of plant secondary metabolites. Light exposing may also stimulate enzyme phenylalanine ammonium lyase (an enzyme which control the production of phenolic compounds) of many plant tissues to influence the production of SPD (Sahai and Shuler 1984). According to Sahai (1994) the production of secondary metabolite such as sesquiterpenes in callus culture of *Chamomilla matricaria* was remarkably stimulated by light.

Effect of incubation temperature

The effect of incubation temperature on the cell growth and SPD production is shown in Figure 4. The highest cell dry weight was obtained at 25 °C (8.415 ± 0.079 g/litre) and 23 °C (7.58 ± 0.029 g/litre) as compared to 15 °C (5.03 ± 0.019 g/litre) and 31 °C (5.68 ± 0.039 g/litre) after 30 days of growth. However, the production of SPD was higher at 25 °C ($3.37 \mu\text{g/g dw}$) and 31 °C ($2.24 \mu\text{g/g dw}$) as compared to 15 °C ($0.55 \mu\text{g/g dw}$) and 23 °C ($0.848 \mu\text{g/g dw}$). In the present study, incubation of *G. umbrosus* cell culture at the lower temperature (15 and 23 °C) inhibited callus growth and SPD production completely. However, the cell incubation at 31 °C reduced cell growth but did not affect the accumulation of SPD. This suggested that,

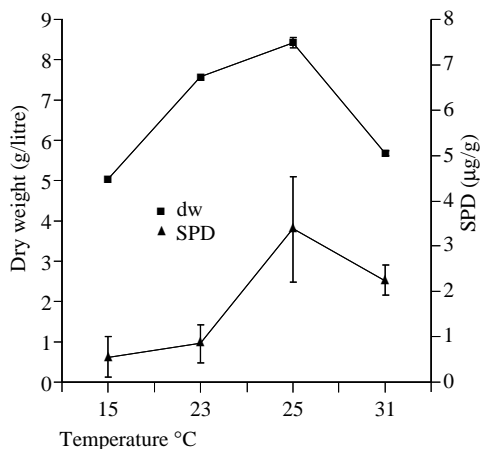


Figure 4. Effect of temperature on growth (dw) and SPD production of *Goniothalamus umbrosus* callus culture. Bar indicates standard error of mean ($n = 3$)

changes in temperature affected the cell growth and the SPD production.

The study observed that the optimum temperature for better cell growth and SPD production was 25 ± 2 °C. Previous study by Imam (1998) showed that the maximum cell growth of *G. umbrosus* cell culture was also at 25 ± 2 °C. According to Endress (1994), the optimum temperature for secondary metabolite production is usually different from that for culture growth which is usually between 20 °C and 25 °C. Abdullah et al. (1998) reported that the incubation at 25 °C and 27 °C showed better cell growth and anthraquinone content in cell suspension culture of *Morinda elliptica*. Low temperature slows the growth and lowers the cell dry weight of culture because according to Endress (1994), low temperatures tend to compensate the inhibiting effect of hormone (auxin) as an important part of cell culture growth (2,4-D). The SPD contents were significantly different at 25 °C and 30 °C compared to 15 °C and 23 °C. Temperature that deviates too far from the average tends to cause stress and usually has an activating effect on secondary metabolite production (Endress 1994).

Conclusion

The optimum cell growth and SPD content in callus culture of *G. umbrosus* was obtained in MS medium supplemented with 3% sucrose, 3.0 mg/litre of 2,4-D and 0.5 mg/litre of BAP under light condition with incubation temperature at 25 ± 2 °C. The maximum growth of callus culture obtained was 31.75 ± 2.25 g fw/culture and 4.73 ± 0.18 g dw/culture after 6 weeks of incubation and the SPD accumulation in callus varied from 0.041–1.96 µg/g sample.

References

- Abdullah, M.A., Ali, A.M., Marziah, M., Lajis, N.H. and Ariff, A.B. (1998). Establishment of cell suspension culture of *Morinda elliptica* for the production of antraquinones. *Plant Cell, Tissue and Organ Culture* 54: 173–82
- Azimahtol, H.L.P. and Johnson, S. (1998). Antagonistic effects of styrylpyrone derivative (SPD) on 7, 12-Dimethylbenzanthracene-induced rat mammary tumors. *In vivo* 12: 403–10
- Azimahtol, H.L.P. and Radhakrishnan, S. (1994). Mode of action of natural anticancer compound: Styrylpyrone derivative from *Goniothalamus* spp. *Proceedings of Third Malaysian Society of Applied Biology Symposium*: 19–20 June 1994, Melaka, p. 103–5. Melaka: UKM
- Eapen, S. and George, L. (1990). Ontogeny of somatic embryos of *Vigna aconitifolia*, *V. mungo* and *V. radiata*. *Ann. Bot.* 66: 219–26
- Endress, R. (1994). *Plant Cell Biotechnology*. Berlin: Springer-Verlag
- Gibson, D.M., Ketchum, R.E.B., Vance, N.C. and Christen, A.A. (1993). Initiation and growth of cell lines of *Taxus brevifolia* (Pacific yew). *Plant Cell Report* 12: 479–82
- Imam, M. (1998). Pengujian kaedah in vitro pembiakan *Goniothalamus umbrosus* J. Sinclair dan kesannya terhadap kandungan goniotalamine. Tesis Sarjana Sains. Fakulti Sains Hayat, UKM, Bangi
- Jewers, K., Davis, B., Dougan, J. and Wetchapinan, S. (1972). Goniothalamine and its distribution in four *Goniothalamus* species. *Phytochemistry* II: 2025–30
- Maretzki, A. Thom, M. and Nickell, L.G. (1974). Utilization and metabolism of carbohydrates in cell and callus culture. In: *Tissue Culture and Plant Science* (Street, H.E., ed.), p. 329–62. London, New York: Academic press
- Rokem, J.S. and Goldberg, I. (1985). Secondary metabolites from plant cell suspensions: methods for yields improvement. *Adv. In Biotechnology Processes* 4: 241–71
- Sahai, O.M. (1994). Plant tissue culture. In: *Bioprocess Production of Flavour and Fragrance and Color Ingredients* (Gabelman, A., ed.), p. 239–74. Singapore: John Wiley and Son
- Sahai, O.M. and Shuler, M. (1984). Environmental parameters influencing phenolic production by batch culture of *Nicotiana tabacum*. *Biotechnol. Bioeng.* 26: 111–20
- Schoofs, G.S., Tieichmann, S., Hartman, T. and Wink, M. (1983). Lysine decarboxylase in plant and its interaction in quinolizidine alkaloid biosynthesis. *Phytochem.* 22: 65–9
- Zenk, M.H., El-Shagi, H. and Schulte, U. (1975). Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. *Planta Medica*. 29: 79–101

Abstrak

Pengkulturan tisu *Goniothalamus umbrosus* merupakan kaedah alternatif penghasilan bahan metabolit terbitan stirilpiron (SPD). Kajian ini dilakukan untuk mengoptimumkan pertumbuhan sel dan penghasilan SPD. Kadar pertumbuhan kultur diukur berdasarkan berat basah (bb) dan kering (bk) pada sela masa tertentu manakala kandungan SPD ditentukan dengan menggunakan Kromatografi Cecair Prestasi Tinggi (HPLC). Kadar pertumbuhan dan penghasilan SPD yang optimum dihasilkan melalui pengkulturan kalus di dalam medium Murashige & Skoog (MS) yang ditambah dengan 3% sukrosa dan kombinasi hormon 3.0 mg/liter 2,4-D dan 0.5 mg/liter benzyl amino purine (BAP) pada persekitaran bercahaya terang dan suhu 25 ± 2 °C. Pertumbuhan maksimum kalus ialah 31.75 ± 2.25 g/liter bb/kultur dan 4.73 ± 0.18 g/liter bk/kultur pada 6 minggu pengkulturan. Analisis HPLC menunjukkan kandungan SPD di dalam kalus ialah 0.041–1.96 µg/g bk sampel.