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RESEARCH PAPER

Solasodine Production from Cell Culture of *Solanum hainanense* Hance

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Abstract Stem explants of *Solanum hainanense* Hance plantlets were cultured on Murashige and Skoog solid medium, containing 3% (w/v) sucrose, supplemented with 0.1 mg/L benzylaminopurine (BAP) and 1.0 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) for callus production. To establish the cell suspension culture, 3 g of fresh callus were cultured in 50 mL of the same medium, but without a solid agent, at an agitation speed of 120 rpm. Every 15 mL of culture was sub-cultured in fresh MS liquid medium for maintenance. The cell biomass of *S. hainanense* reached a maximum value of 18.47 g after 4 weeks of culture on the same MS medium, but with the sucrose content increased to 4%, at an agitation speed of 150 rpm, with 20 mL of inoculum. Analysis via high performance liquid chromatography (HPLC) showed that the solasodine content in the cell suspension after 4-weeks old (121.01 mg/g) was higher than that of *in planta* 1-year old roots (20.52 mg/g) by approximately 6-fold.

Keywords: callus, cell suspension, solasodine, HPLC, *Solanum hainanense*

1. Introduction

S. hainanense Hance, a member of the Solanaceae family, is a valuable medicinal plant found in many areas of China and Vietnam, and has been used as an anti-inflammatory, anti-histaminemia and against vernomous snakes [1].

Plants of *S. hainanense* accumulate glycoalkaloid mainly in their roots [2]. Glycoalkaloids are secondary plant metabolites, also known as phytoalexins [3]. Although they have been reported to be potentially toxic, glycoalkaloids and their hydrolysis products, without the carbohydrate side chain, also have beneficial effects. However, this plant has never been cultivated, and its various properties, including seasonal variation in growth and production of glycoalkaloid, remain to be studied. Glycoalkaloids have been found to inactivate the *Herpes simplex*, *H. zoster*, and *H. genitalis* viruses in humans [4], protect mice against infection by *Salmonella typhimurium* [5], enhance the duration of the action of anesthetics, which act by inhibiting acetylcholinesterase [6], lower cholesterol [7,8] and serve as a malaria vaccine [9].

Several papers have described the production of solasodine, a type of glycoalkaloid, in cell and tissue cultures of *S. laciniatum*, which has been reviewed by Macek [10], *S. nigrum* [11], *S. aviculare* [12,13], *S. khasianum* [14], *S. lyratum* [15], and *S. eleagnifolium* [16]. However, solasodine production in the cultured tissue of *S. hainanense* has received little attention, with only a limited number of reports relating to the callus culture of *S. hainanense* [17]. Thus, the objective of this research was to establish a *S. hainanense* cell suspension culture and investigate the accumulation of solasodine in these cells.

2. Materials and Methods

2.1. Callus culture

Stem segment explants, 0.5 cm in length, were excised from *in vitro S. hainanense* plants grown on the MS medium [18], supplemented with 3% (w/v) sucrose, 0.5 mg/L indolylbutyric acid (IBA), and 0.8% (w/v) agar. The explants were then placed on the MS medium, supplemented with 3% (w/v) sucrose, 0.5 ~ 1.5 mg/L 2, 4-dichorophenoxyacetic acid (2, 4-D), 0.1 ~ 1.5 mg/L benzylaminopurine (BAP), 0.5 ~ 2.0 mg/L kinetin, and 1.0 mg/L naphthalene acetic acid (NAA), with 0.8% (w/v) agar for callus induc-

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tion. The pH of the medium was adjusted to 5.8, and then autoclaved at 121°C for 15 min. The cultures were incubated at 25 ± 2°C under a light intensity of 2,000 ~ 3,000 lux, with a photoperiod of 10-h day light.

2.2. Establishment of cell suspension culture

Cell suspension cultures were initiated *via* the agitation of 3 g of callus in 250 mL Erlenmeyer flasks, containing 50 mL of MS liquid medium, supplemented with 1.0 mg/L 2, 4-D and 0.1 mg/L BAP, at 120 rpm for 3 weeks, under the same conditions as for the callus culture, with the exception of a light intensity of 500 lux, until a suspension of free cells formed. In order to maintain the suspension culture, every 15 mL (approximately 1.25 g fresh cell biomass) of 3-week old culture was transferred to fresh MS liquid medium.

Samples were obtained every week to determine both the fresh and dry weight cell biomass under the influence of sucrose contents (1 ~ 5%, w/v), agitation speeds (80 ~ 180 rpm) and inoculum sizes (5 ~ 20 mL). To measure the fresh cell weight, the cells in the suspension culture were filtered, washed with distilled water, collected and weighed. The dry cell weight was determined by drying the fresh cell biomass at 40°C to a constant weight (approximately 24 h).

Growth index = Final fresh cell weight/Initial inoculums fresh cell weight.

2.3. Quantification of solasodine

Dry cell biomass of *S. hainanense* was ground to a fine powder. One gram of this powder was pretreated *via* Soxhlet extraction (J. P. Selecta, Spain), with a mixture of acetic acid : methanol (5:95, v/v) for 3 h. The extract was then filtered and completely concentrated at 50°C using a vacuum rotary concentrator (Heidolph, Germany). The concentrate was dissolved by methanol to 10 mL (solasodine extract), filtered through Minisart 0.45 μm membranes (Sartorius, Germany), diluted 5-fold and subjected to high performance liquid chromatography (HPLC) at ambient temperature, using a Vertisep GES C18 column (5 μm, 4.6 × 150 mm), a flow rate of 1 mL/min, run time of 10 min, detector wavelength of 254 nm, stationary phase of silica gel (reverse phase) and a mobile phase of 100% methanol. Fifty microliters of sample was injected onto the column using a Hamilton syringe.

The high performance liquid chromatography analysis was performed on a LC-20A Prominence system (Shimadzu, Japan), with LC-Solution software. All solvents were of analytical grade and purchased from Sigma and Merck & Co., Inc.

A solasodine solution (0.5 mg/mL in methanol), from Sigma, was used as a standard for determination of the solasodine concentration.

2.4. Statistical analysis

All experiments were performed in triplicate. The data were analyzed in terms of the means ± standard error, followed by comparisons of the mean *via* Duncan's tests, at $p < 0.05$, using the SAS program (ver. 6.12).

3. Results and Discussion

3.1. Callus culture

As shown in Table 1, the effects of plant growth regulators (PGRs) on the callus formation were investigated using in vitro stem segments of *S. hainanense* plants. The MS medium, supplemented with 1.0 mg/L 2, 4-D and 0.1 mg/L BAP, showed the strongest induction; calli were yellow in color, compact and friable after 4 weeks of culture (Fig. 1A). The other combinations of PGRs resulted in

Table 1. Effects of plant growth regulators on callus induction and morphogenesis

PGR (mg/L)				Callus induction	Callus morphogenesis
2,4-D	NAA	BAP	Kinetin		
0.5	–	–	0.5	++	Light yellow and soft
1.0	–	–	0.5	+++	Light yellow and soft
1.5	–	–	0.5	++	White and soft
–	1.0	–	1.0	++	White and soft
–	1.0	–	1.5	+	Light green and soft
–	1.0	–	2.0	+	Light green and soft
1.0	–	0.1	–	++++	Yellow, compact and friable
1.0	–	0.5	–	+++	Yellow, compact and friable
1.0	–	1.0	–	+++	White and soft
–	1.0	1.0	–	+	Light green and soft
–	1.0	1.5	–	++	Light green and soft
–	1.0	2.0	–	+	Light green and soft

+: induction, ++: low production of callus; +++: medium production of callus; ++++: high production of callus.

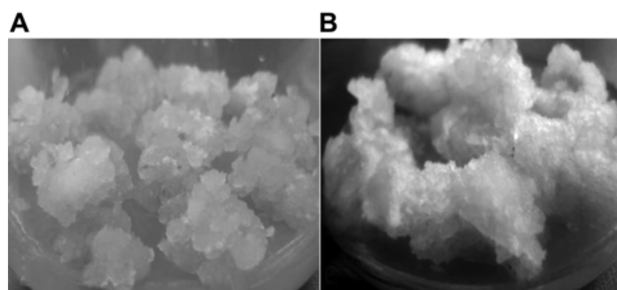

Fig. 1. Calli of *S. hainanense*. A: MS medium with 0.1 mg/L BAP and 1.0 mg/L 2, 4-D, B: MS medium with 1.0 mg/L NAA and 1.0 mg/L 2, 4-D.

poor induction; calli could be white, light green or light yellow in color, and soft (Fig. 1B); or no-induction (data not shown). It was also found that medium supplemented with 1.0 mg/L 2, 4-D in combination with 0.1 ~ 1.0 mg/L BAP had significant effects on callus formation. Primary calli (yellow in color, compact and friable) were transferred onto MS medium, supplemented with 1.0 mg/L 2, 4-D and 0.1 mg/L BAP, for proliferation. The secondary calli obtained after 4 weeks of culture were sub-cultured and maintained in fresh medium with the same composition. Anh *et al.* [17] found that leaf explants of *S. hainanense* plantlets strongly induced callus on MS medium, supplemented with 0.5 mg/L kinetin and 1.0 mg/L 2, 4-D. However, these calli could not develop a cell suspension.

3.2. Cell suspension culture

A suspension culture was established from secondary callus to investigate the biomass accumulation of *S. hainanense* cells. Approximately a 3 g fresh weight of callus was transferred to 50 mL of MS liquid medium, supplemented with 1.0 mg/L 2, 4-D and 0.1 mg/L BAP. Suspension cells were initially generated, as shown in Fig. 2 within 3 weeks of culture.

The growth of cells was determined via fresh and dry weight measurements. The fresh and dry cell weights were recorded every week for 7 weeks of culturing. It was observed that the cell biomass increased with increasing culture time from weeks 1 to 4, with a maximum value of 10.36 g fresh weight (approximately 0.44 g dry weight). Typical cell growth curves (Fig. 3) showed an exponential growth phase lasting approximately 4 weeks, and ended at the death phase (weeks 5 ~ 7). The lag and stationary phases possibly only occurred in several days; therefore, they were difficult to observe. The entire growth curve took approximately 7 weeks to complete, and presented more than a 3-fold accumulation of fresh cell biomass.

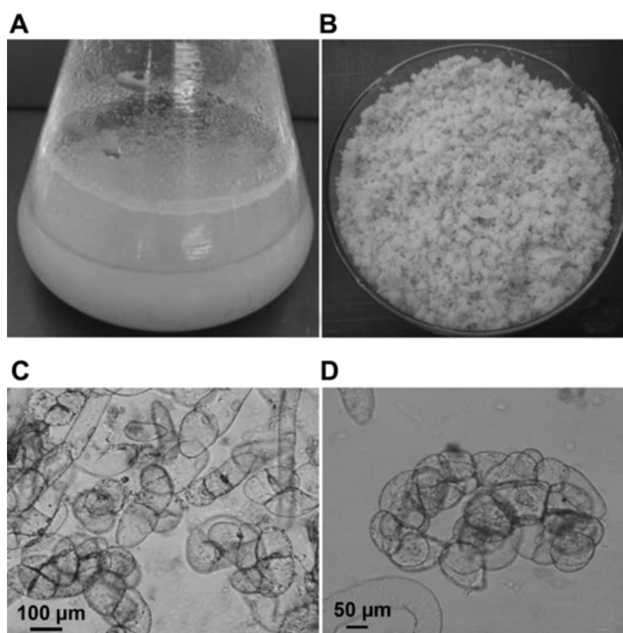

Fig. 2. Cells of *S. hainanense*. A: cell suspension, B: fresh cell biomass, C and D: cells under microscope.

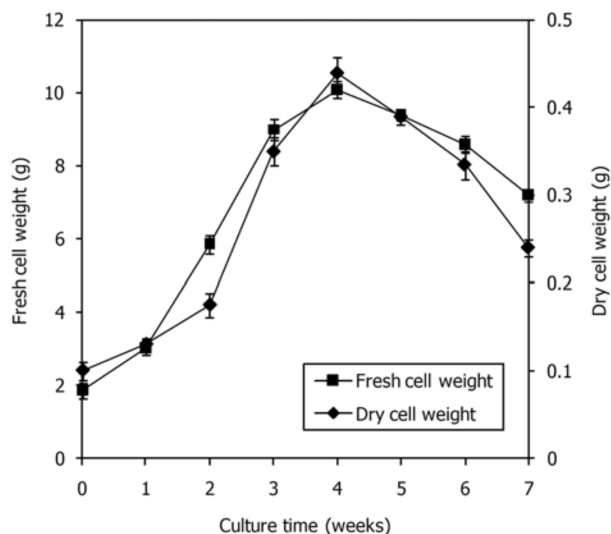

Fig. 3. Biomass production of *S. hainanense* cells in a batch culture ($p < 0.05$).

3.2.1. *Effect of sucrose on cell growth*

The cell biomass, under the effect of different sucrose contents between 1 and 5% (w/v), was investigated after the 4th week of culture (Table 2). The highest fresh and dry weights were attained in medium containing 4% sucrose. Based on the cell growth index, the cell fresh weight increased 13.40-fold in the presence of 4% sucrose. However, despite sucrose being an important carbon and energy source, increasing its concentration to 5% resulted in a reduction of the fresh cell weight of approximately 15% compared with that of 4% sucrose. From the results of this experiment, a sucrose content of 4% was chosen for the next experiments.

Akalezi *et al.* [19] investigated *Panax ginseng* cell growth under various initial sucrose concentrations, and obtained a

Table 2. Effect of sucrose content on cell biomass accumulation

Sucrose content (%)	Fresh cell weight (g)	Dry cell weight (g)	Growth index
1	8.94^{d}	0.22^{d}	7.15
2	10.36^{c}	0.55^{c}	8.29
3	15.05^{b}	0.62^{c}	12.04
4	16.75^{a}	0.92^{a}	13.40
5	14.11^{b}	0.80^{b}	11.29

Different letters indicate significantly different means *via* a Duncan's test ($p < 0.05$).

maximum cell yield of 0.83 with an initial sucrose level of 30 g/L. Lee *et al*. [20] indicated that the *Gymnema sylvestre* cell growth index reached its highest value of 9.64 in medium containing 3% sucrose. Another report on the effect of the sucrose concentration (2 ~ 9%) on the growth of a suspension compact callus cluster (CCC) of *Catharanthus roseus* showed a highest biomass accumulation of 14.6 ~ 14.8 g dry weight/L at 40 ~ 50 g/L sucrose [21].

3.2.2. Effect of agitation speed on cell growth

The effects of agitation speeds between 80 and 180 rpm on the growth of *S. hainanense* cells are shown in Table 3. Cells reached a maximum biomass concentration of 17.47 g fresh weight (approximately 0.98 g dry weight with growth index of 13.98) after 4 weeks of culturing at 150 rpm. The growth of *S. hainanense* cells was reduced with increasing agitation speed up to 180 rpm, where the cell biomass remained at 15.81 g fresh weight, with a growth index of 12.65. The growth and proliferation of the cells in flask cultures was found to depend on agitation for mixing to prevent biomass sedimentation. However, if the agitation speed was too strong, this might cause the cell walls to breakdown, with the accumulation of cell debris. From the results of this experiment, an agitation speed of 150 rpm was chosen for the next experiments.

In our other work [22], the effects of agitation speed (80 ~ 150 rpm) on *Centella asiatica* cell growth was also investigated. Cells reached their highest growth index of 4.29 after 24 days of culturing at 120 rpm. However, Zhao *et al*. [21] showed that the shaking speed (90 ~ –150 rpm) had no significant affect on the biomass accumulation of the CCC cultures of *Catharanthus roseus*.

3.2.3. Effect of inoculum size on cell growth

The effect of the inoculum size on cell biomass production was investigated by transferring between 5 and 20 mL of the shaking flask culture at 3-weeks old into fresh medium. The results are shown in Table 4, which indicated a volume of 20 mL of cultured cells reached a maximum fresh biomass of 18.47 g (1.29 g dry weight), with a growth index of 14.78 after 4 weeks of culturing, which was much more than that obtained using the other volumes (3.15 ~ 16.52 g fresh weight/0.21 ~ 0.89 g dry weight/2.52 ~ 13.22 growth index). Thus, the inoculum size was observed to have a significant influence on the growth of a *S. hainanense* suspension culture, with fresh and dry cell weights increasing significantly. From the results of this experiment, a sucrose content of 4%, agitation speed of 150 rpm and inoculum size of 20 mL were chosen to investigate the accumulation of solasodine.

Table 3. Effect of agitation speed on cell biomass accumulation

Agitation speed (rpm)	Fresh cell weight (g)	Dry cell weight (g)	Growth index
80	7.25[d]	0.39[c]	5.80
100	9.24[c]	0.38[c]	7.39
120	16.34[b]	0.82[b]	13.07
150	17.47[a]	0.98[a]	13.98
180	15.81[b]	0.79[b]	12.65

Table 4. Effect of inoculum size on cell biomass accumulation

Inoculum size (mL)	Fresh cell weight (g)	Dry cell weight (g)	Growth index
5	3.15[d]	0.21[c]	2.52
10	7.81[c]	0.35[c]	6.25
15	16.52[b]	0.89[b]	13.22
20	18.47[a]	1.29[a]	14.78

According to Akalezi *et al*. [19], *Panax ginseng* cell growth was low with a small inoculum size of 1.5 g dry weight/L, with the maximum cell growth rate obtained with 3 g dry weight/L of inoculum size. Lee *et al*. [20] showed that inoculum sizes (20 ~ 120 g/L) also had a significant influence on the growth profile of *G. sylvestre* suspension cultures, with the growth rate increasing significantly when the initial inoculum was 60 g/L.

3.3. Quantification of solasodine

The solasodine concentration in the cells was investigated by HPLC from weeks 1 to 7 of culturing, which showed that the culture of *S. hainanense* cells produced solasodine.

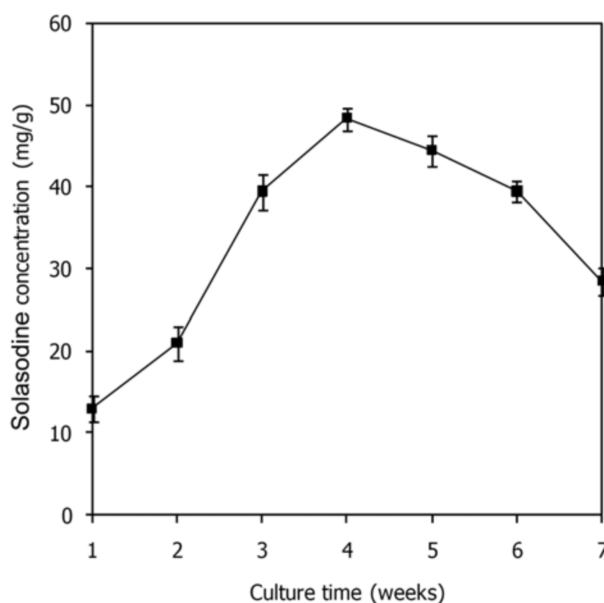

Fig. 4. Dynamics of solasodine accumulation in cells of *S. hainanense*.

Table 5. Solasodine concentration

Explants	Retention time (min)	Peak area $(mAU)^2$	Solasodine concentration
Solasodine	2.02	3,071,728	0.50 mg/mL
Suspension cells	1.96	14,868,713	121.01 mg/g
In planta root	1.98	2,521,630	20.52 mg/g

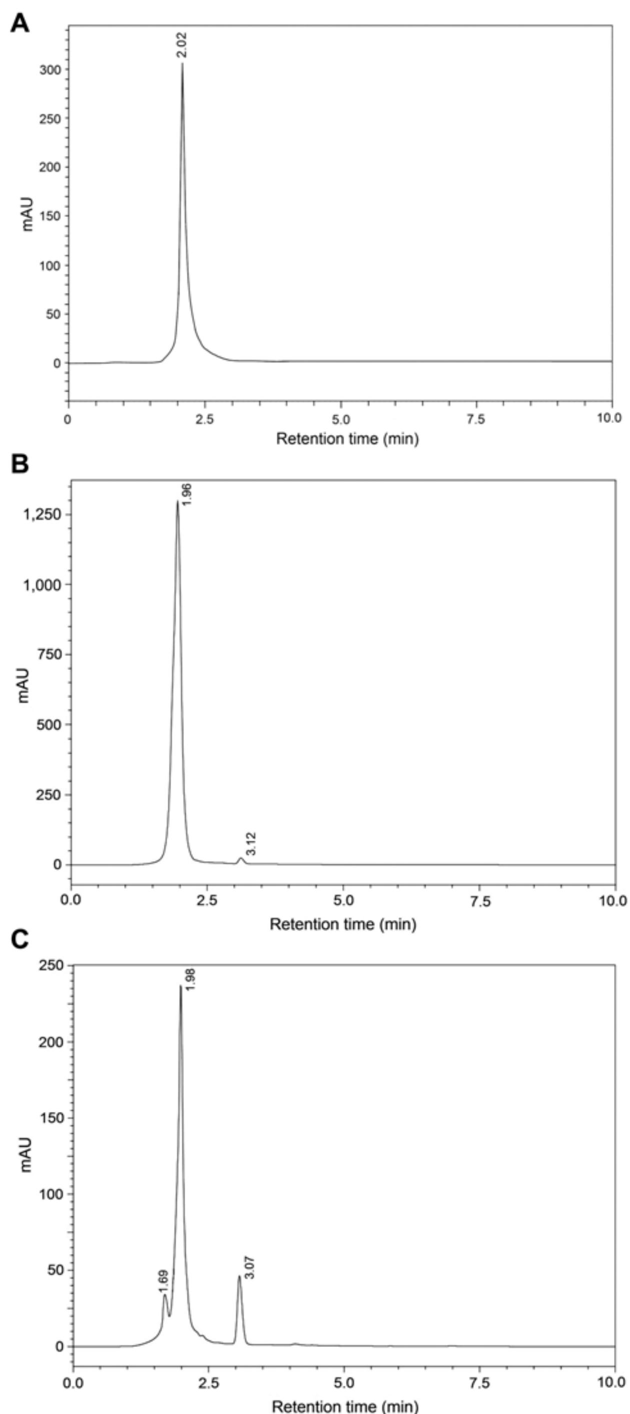

Fig. 5. HPLC chromatogram of solasodine: A: standard solasodine (0.5 mg/mL), B: solasodine extract from suspension cells, and C: solasodine extract from *in planta* root.

Fig. 4 illustrates the profile of solasodine accumulation as a function of the culture time on MS medium, supplemented with 0.1 mg/L BAP and 1.0 mg/L 2, 4-D. The accumulation of solasodine reached a maximum value of 121.01 mg/g after 4 weeks of culture in optimal condition (4% sucrose, 150 rpm agitation speed and 20 mL inoculum size); whereas, our study also showed a solasodine concentration from *in planta* root (1 year old) of only 20.52 mg/g. A Duncan's test ($p < 0.05$) on the mean confirmed that these samples were significantly different (Table 5). These results showed that these cells are suitable for the production of solasodine by *S. hainanense*. The results from earlier experiments [23] revealed a total glycoalkaloid concentration per dry weight of less than 0.2% in the roots when this plant species was grown under natural condition.

As shown in Figs. 5A, 5B, and 5C, the HPLC chromatograms indicated that the solasodine produced a peak with a retention time of 2.02 min; solasodine was also detected in cells grown on a medium supplemented with 1.0 mg/L 2, 4-D and 0.1 mg/L BAP and *in planta* roots with equivalent retention times of 1.96 and 1.98 min, respectively.

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References

1. Thai, N. P., L. V. Trung, N. K. Hai, and L. Huynh (1998) Protective efficacy of *Solanum hainanense* Hance during hepatotoxicity in male mice with prolonged and small oral doses of trinitrotoluene. *J. Occup. Health* 40: 276-278.
2. Hop, V. V. and V. X. Phuong (2003) Alkaloid species belong to Solanaceae family in Vietnam. *Vietnamese J. Biol.* 25: 27-31.
3. Chating, B., L. Conepcion, B. de Cristancho, and A. Usubillaga (1997) Estudio clinico de la efectividad de extractos alcaloides optenidos de los frutos del *Solanum americanum* Miller sorbe el *Herpes simplex*, *Herpes zoster* y *Herpes genitalis*. *Revista de la Facultad de Farmacia* 32: 18-25.
4. Stanker, L. H., C. Kampos-Holtzapple, R. C. Beier, C. E. Levin, and M. Friedman (1996) Detection and quantification of glycoalkaloids-comparison of enzyme-linked immunosorbent assay and high-performance liquid chromatography methods. *ACS Symp. Ser.* 621: 243-251.
5. Gubarev, M. I., E. Y. Enioutina, J. L. Taylor, D. M. Visic, and R. A. Daynes (1998) Plant-derived glycoalkaloids protect mice against lethal infection with *Salmonella typhimurium*. *Phytother. Res.* 12: 79-88.
6. McGehee, S., D. Kraswoski, and L. Fung (2000) Cholinesterase inhibition by potato glycoalkaloids slows mivacurium metabolism. *Anesestesiol.* 93: 510-519.
7. Friedman, M., T. E. Fitch, C. E. Levin, and W. H. Yokoyama (2000) Feeding tomatoes to hamsters reduces their plasma low-density lipoprotein cholesterol and triglycerides. *J. Food Sci.* 65:

897-900.
8. Friedman, M., T. E. Fitch, and W. H. Yokoyama (2000) Lowering of plasma LDL cholesterol in hamsters by the tomato glycoalkaloid tomatine. *Food Chem. Toxicol*. 38: 549-553.
9. Heal, K. G., N. A. Sheikh, M. R. Hollingdale, W. J. W. Morrow, and A. W. Taylor-Robinson (2001) Potentiation by a novel alkaloid glycoside adjuvant of a protective cytotoxic T cell immune response specific for a preerythrocytic malaria vaccine candidate antigen. *Vaccine* 19: 4153-4161.
10. Macek, T. E. (1989) *Solanum aviculare* Forst, *Solanum laciniatum* Ait: *In vitro* culture and the production of solasodine. pp 443-463. In: Y. P. S. Bajaj (ed.). *Biotechnology in agriculture and forestry.* Springer Verlag, Berlin Heidelberg.
11. Yogananth, N., R. Bhakyaraj, A. Chanthuru, S. Parvathi, and S. Palanivel (2009) Comparative analysis of solasodine from *in vitro* and *in vivo* cultures of *Solanum nigrum* Linn. *Kathmandu Univ. J. Sci. Engin. Technol*. 5: 99-103.
12. Kittipongpatana, N., R. S. Hock, and J. R. Porter (1998) Production of solasodine by hairy root, callus, and cell suspension cultures of *Solanum aviculare* Forst. *Plant Cell Tiss. Org. Cult*. 52: 133-143.
13. Yu, S. X., K. H. Kwok, and P. M. Doran (1996) Effect of sucrose, exogenous product concentration, and other culture conditions on growth and steroidal alkaloid production by *Solanum aviculare* hairy roots. *Enz. Microb. Tech.* 18: 238-243.
14. Jacob, A. and N. Malpathak (2005) Manipulation of MS and B5 components for enhancement of growth and solasodine production in hairy root cultures of *Solanum khasianum* Clarke. *Plant Cell Tiss. Org. Cult*. 80: 247-257.
15. Lee, M. H., J. J. Cheng, C. Y. Lin, Y. J. Chen, and M. K. Lu (2007) Precursor-feeding strategy for the production of solanine, solanidine and solasodine by a cell culture of *Solanum lyratum*. *Proc. Biochem*. 42: 899-903.
16. Nigra, H. M., M. A. Alvarez, and A. M. Giulietti (1990) Effect of carbon and nitrogen sources on growth and solasodine production in batch suspension cultures of *Solanum eleagnifolium* Cav. *Plant Cell Tiss. Org. Cult*. 21: 55-60.
17. Anh, N. H. T., L. T. H. Thanh, D. H. N. Binh, T. T. B. Phuong, and N. H. Loc (2007) Study on the accumulation of glycoalkaloid in *Solanum hainanense* Hance callus. Proc the 2007th National Conference on Life Sciences, Ha Noi, Viet Nam 229-232.
18. Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant* 15: 473-497.
19. Akalezi, C. O., S. Liu, Q. S. Li, J. T. Yu, and J. J. Zhong (1998) Combined effects of initial sucrose concentration and inoculum size on cell growth and ginseng saponin production by suspension cultures of *Panax ginseng*. *Proc. Biochem*. 34: 639-642.
20. Lee, E. J., M. Mobin, E. J. Hahn, and K. Y. Paek (2006) Effects of sucrose, inoculum density, auxins, and aeration volume on cell growth of *Gymnema sylvestre*. *J. Plant Biol.* 49: 427-431.
21. Zhao, J., W. H. Zhu, Q. Hu, and X. W. He (2001) Enhanced indole alkaloid production in suspension compact callus clusters of *Catharanthus roseus*: Impact of plant growth regulators and sucrose. *Plant Growth Regulation* 33: 33-41.
22. Loc, N. H. and N. T. T. An (2010) Asiaticoside production from centella (*Centella asiatica* L. Urban) cell culture. *Biotechnol. Bioproc. Eng.* 15: 1065-1070.
23. Thu, N. B. and P. K. Man (2000) Quantitative determination of glycoalkaloids in *Solanum hainanense* Hance by acid dye colorimetric method. *Vietnamese J. Pharm. Materials* 5: 104-108.