Biotransformation of Hydroquinone to Arbutin by Cell-Suspension Cultures of Three Thai Solanaceous Plants

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ABSTRACT

Cell-suspension cultures of Capsicum annuum L., Solanum aculeatissimum Jacq. and, Datura fastuosa L. (Solanaceae) were established and investigated for the ability to biotransform exogenously-supplied hydroquinone to arbutin. All cultures exhibited good 24-day growth in Murashige and Skoog medium, supplemented with 2,4-dichlorophenoxyacetic acid and benzylaminopurine as plant growth regulators, and with sucrose as a carbon source. The feeding of 15.57 mM hydroquinone into the system resulted in the production of arbutin after 24 hours and reached a maximum value after 2 to 5 days. The cell-suspension culture of C. annuum exhibited the fastest growth and the strongest glycosylation ability. The maximum yield of arbutin, as determined by a HPLC method, was 368.71±57.46 mg/L in the suspension culture of C. annuum. Cell cultures of S. aculeatissimum and D. fastuosa also showed the production of arbutin. For all systems, the amount of arbutin released into the culture medium was higher than that accumulated in the cells.

Key words: arbutin, HPLC, cell suspension culture, biotransformation, Capsicum, Solanum, Datura

INTRODUCTION

Plant cell cultures can serve as a source of enzymes that carry out the biotransformation of chemicals exogenously supplied into the system. This strategy has been used to produce high-value and/or biologically-active phytochemicals from the lower-value substrates (Yokoyama, 1996). The production of a natural tyrosinase inhibitor arbutin (hydroquinone-β-D-glucopyranoside; Figure 1A) from its precursor hydroquinone (Figure 1B) is an example of utilizing plants or plant cells as "chemical factories". The success on arbutin biotransformation by plant cells was first reported by Tabata and co-workers on cell cultures of *Datura inoxia*, which glucosylated hydroquinone into arbutin within 10 hr after administration (Tabata et al., 1976). The efficiency of *D. inoxia* cells to convert hydroquinone into arbutin was later investigated and maximum yields of 4.2 and 7.1 g/L were achieved at the usual cell density and at a high cell density, respectively (Suzuki et al., 1987). In 1991, a

stable, high level production of arbutin from hydroquinone was reported using cell suspension cultures of *Catharanthus roseus*. The arbutin yield was reported to be 9.2 g/L, which was 45% of cell dry weight. Both systems were patented and since then there have been many reports on arbutin production using plant tissue cultures of many plants. Examples included cell cultures of *Bellis perennis, Bergenia crassifolia* (5.10%), *Brassica oleracea* var. *capitata, Coronilla varia, Leonurus cardiaca* (4.80%), *Leuzea carthamoides* (0.90%), *Rheum palmatum, Rhodiola rosea* (6.40%) and *Datura meteloides* (6.60%) (Duskova et al., 1999; Jahodar et al., 1999), *Rauwolfia serpentina* (Lutterbach and Stoeckigt, 1992), *Echinacea purpurea* (4.01%), *Exacum affine* (3.44%), *Melittis melissophyllum* (1.79%), *Ruta graveolens* (2.48%) and *Ruta graveolens ssp. divaricata* (5.07%) (Skrzypczak-Pietraszek et al., 2005), as well as hairy root cultures of *Brugmansia candida* (Casas et al., 1998) and *Panax ginseng* CA Mayer (13%) (Zhao et al., 2001)

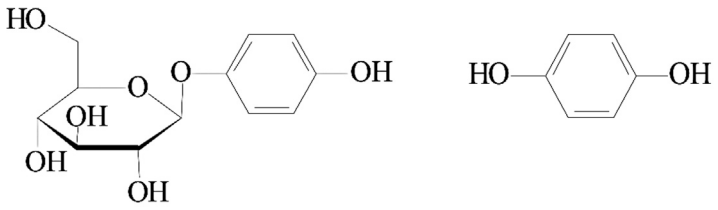


Figure 1. Chemical structures of arbutin [A] and hydroquinone [B].

Despite a continuous search for new arbutin-producing plants and plant cell systems, a survey of literature, to date, shows no report of such plant or system in Thailand. However, a number of Thai medicinal plants, particularly those from the family Solanaceae, are known to contain glycosides as their secondary metabolites, which suggest that these plants may be equipped with enzymes that can carry out glycosylation. In this study, cell suspension cultures of three plants selected as the representative of each genus, *Capsicum annuum, Datura fastuosa* and *Solanum aculeatissimum* were investigated for the ability to biotransform hydroquinone to arbutin. An isocratic HPLC condition developed by Kittipongpatana et al., (2006) was employed in the detection and quantitation of arbutin in the liquid medium samples.

MATERIALS AND METHODS

Materials

Chemicals

Arbutin standard (>98%) and medium salt base were purchased from Sigma Chemical Co. (St. Louis, MO). Hydroquinone was supplied by Fischer Chemicals. Other medium components and plant growth regulators (PGRs) were of tissue culture grade or equivalent. All solvents used for chromatographic purposes were of HPLC

grade. Other solvents were of reagent grade or equivalent.

Plant Materials

The seeds of *Capsicum annuum* L. and *Solanum aculeatissimum* Jacq. were obtained from commercial sources. The seeds of Datura fastuosa L. were obtained from the medicinal plant garden of the Faculty of Pharmacy, Chiang Mai University. Voucher specimens are deposited in the herbarium of the Faculty of Pharmacy, Chiang Mai University. The seeds were surface-sterilized with 20% v/v sodium hypochlorite (Clorox®) solution with Tween® 80 as wetting agent for 15 to 20 min. After rinsing with sterile distilled water (three times, 2-3 minutes each), seeds were germinated on hormone-free medium, containing one-quarter strength Murashige and Skoog (MS) salt base and 2% w/v sucrose. The seedlings were grown in sterile vessels at 25°C with a 16/8 hours light/dark cycle (light intensity 300 μmol $m^{-2}s^{-1}$ PPFD, 100% cool-white fluorescent).

Methods

Establishment of Callus and Cell-Suspension Cultures

• Callus Cultures

Callus tissues of all three plants were initiated by placing sterile leaf or stem explants (2-3 cm long), excised from three-week-old plants, in test tubes containing 10 mL MS medium (2% sucrose) supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene acetic acid (NAA) at three different concentrations (0.5, 1.0 and 2.0 mg/mL). These test tubes were maintained at 25°C with a 16/8 hours light/dark cycle (light intensity 300 μmol m-2s-1 PPFD, 100% cool-white fluorescent). The formation of callus after two weeks from the explants and the appearance of the callus were observed and recorded. The growth of callus was then tested in MS medium, containing different combinations and concentrations of auxin and cytokinin. Callus tissues were maintained by subculturing onto fresh medium every three to five weeks. The medium which yielded the best growth for each plant was selected for use in the cell suspension culture.

• Cell-Suspension Cultures

Cell-suspension cultures were established by inoculating friable callus tissues into a 1-L Erlenmeyer flask containing MS liquid medium, supplemented with 2% sucrose and the selected combination of PGRs. The flask was placed on a shaker, gyrating at 120 rpm for a few days or until a homogenous suspension culture was obtained. For the experiment, 5 mL of established cell suspension was transferred into each of five 200-mL culture bottles. The volume of each bottle was adjusted to 35 mL with the liquid medium. The culture bottles were shaken at 120 rpm. After two weeks of growth, the cultures were used in the biotransformation experiment.

Growth Determination of Cell-Suspension Cultures

The growth of the cell-suspension cultures was determined from the growth curves, plotted between the settled cell volume (SCV) and the age of the cultures.

Biotransformation of Hydroquinone to Arbutin

One mL of 60 mg/mL hydroquinone solution was added into the two-week-old cell-suspension cultures (35 mL) of the plants studied (15.57 mM). The untreated cell suspension cultures were used as controls. The cell-suspension samples were harvested on day 1, 2, 5 and 7 after the addition of hydroquinone.

Analysis of Arbutin

The harvested cells and the culture media were filtered through a Whatman no.1 filter paper to separate the cells from the liquid media. The liquid media were adjusted to 35 mL with distilled water, passed through a 0.45 µm membrane filter into HPLC sample vials. The cells were subjected to several freeze-thaw cycles and finally were crushed and mixed well with purified water. The aqueous extracts were then filtered through a 0.45 µm syringe filter and placed in sample vials. HPLC was performed using a method described by Kittipongpatana et al. (2006). The system (Hewlett Packard HP 1100 LC) consisted of a double-piston pump, an autosampler and a variable wavelength UV/VIS detector. The HPLC column was Apollo C-18 (4.6 x 150 mm, 5 µm particle diameter, 100 Å average pore size) (Alltech Associates, Inc., USA). The methanol: water (90:10) mobile phase was pumped through the column at a flow rate of 0.9 mL/min. Arbutin and hydroquinone were both detected by UV absorption at 280 nm. Each injection volume was 10 µl. The standard solutions of arbutin and hydroquinone were prepared in deionized water. The analysis was done in triplicate.

RESULTS AND DISCUSSION

Establishment and Growth of Tissue Cultures

Callus Cultures

• *Initiation*

Media that contained 2,4-D as auxin promoted formation and growth of callus tissues while the use of NAA resulted in a slower callus formation together with the development of roots. The optimum concentration of 2,4-D for callus formation was between 0.5 to 1.0 mg/l, depending on the plant species (Table 1). Different plants formed callus with different morphology and color. For example, D. fastuosa formed green, friable (GF) callus while C. annuum yielded light brown, friable (LBF) callus and S. aculeatissimum formed green, dense (GD) callus. All plants used in this study could form callus from all parts (leaf, petiole, stem) within two weeks.

Table 1. Formation and morphology of calluses of *C. annuum, D. fastuosa* and *S. aculeatissimum* initiated on MS media containing different types and amounts of auxin. (The results were observed after two weeks).

Plant	Formation* and Morphology** of Calluses on MS medium					
	0.5 mg/l 2,4-D	**1.0 mg/l 2,4-D**	**2.0 mg/l 2,4-D**	**0.5 mg/l NAA**	**1.0 mg/l NAA**	**2.0 mg/l NAA**
C. annuum	++ LBF	+++ LBF	+ LBF	-	+ LBF	+ BF with roots
D. fastuosa	+++ GF	++ GF	++ BF	+ BF with roots	++ BF with roots	++ BF with roots
S. aculeatissimum	++ GD	+++ GD-GF	+ BF	+ GF with roots	+ mostly roots	-

* +++ good ++ moderate + little - none

** GF - green, friable; GD - green, dense; BF - brown, friable; LBF - light brown, friable

Growth of Callus

Two selected concentrations of 2,4-D (0.5 and 1.0 mg/l) were coupled with three levels of BAP (0, 0.1 and 0.5 mg/l). The results of varying the combination of PGRs on callus growth are shown in Table 2. Overall, callus tissues of plants used in this study showed good growth in media containing high ratio of auxin/cytokinin, i.e., 0.5 mg/l 2,4-D + 0.1 mg/l BAP (5:1) and 1.0 mg/l 2,4-D + 0.1 mg/l BAP (10:1). The media with 0.5 or 1.0 mg/l 2,4-D but no BAP showed moderate callus growth, while those with higher cytokinin, i.e., 0.5 mg/l 2,4-D + 0.5 mg/l BAP (1:1) and 1.0 mg/l 2,4-D + 0.5 mg/l BAP (2:1) yielded slow-growing callus tissues compared to the first group. The optimum PGRs combinations in the media for each plant species were 1.0 mg/l 2,4-D + 0.1 mg/l BAP for *C. annuum* and *D. fastuosa*, and 0.5 mg/l 2,4-D + 0.1 mg/l BAP for *S. aculeatissimum*.

Table 2. Growth comparison of *C. annuum, D. fastuosa* and *S. aculeatissimum* calluses cultured on MS media containing different concentrations of 2,4-D and BAP.

Plants	Growth of callus tissues after 2 weeks*					
	0.5 mg/l 2,4-D no BAP	**0.5 mg/l 2,4-D 0.1 mg/l BAP**	**0.5 mg/l 2,4-D 0.5 mg/l BAP**	**1.0 mg/l 2,4-D no BAP**	**1.0 mg/l 2,4-D 0.1 mg/l BAP**	**1.0 mg/l 2,4-D 0.5 mg/l BAP**
C. annuum	++	+++	+	++	++++	++
D. fastuosa	++	++++	++	++	++++	+
S. aculeatissimum	++	++++	++	++	++	+

* ++++ very fast; +++ fast; ++ moderate; + slow; - no growth

Cell Suspension Cultures

The growth of cell suspension cultures was observed by measuring the settled cell volume (SCV) of each of five suspension cultures twice a week over the period of 24 days. A growth curve (Figure 2) was established to determine the growth characteristics and growth pattern of the cultures. Cell suspension culture system of *C. annuum* exhibited the highest growth rate among the plants studied. The "lag" phase was from day 0 to day 5 during which only little growth was observed. The "log" phase, a dramatic increase in cell growth, occurred during day 5 to day 15. After 15 days, the cell growth stabilized or slightly decreased. The final SCV of *C. annuum* cell-suspension cultures was approximately four times higher than the initial SCV and, overall, was significantly higher than those of other plant species. Cell suspension cultures of *D. fastuosa* showed similar growth which reached 3 times of the original SCV after 3 weeks, while the growth of *S. aculeatissimum* cell-suspension cultures was slow in the first 10 days but gradually increased to reach a maximum of 3 times of the initial SCV on day 24.

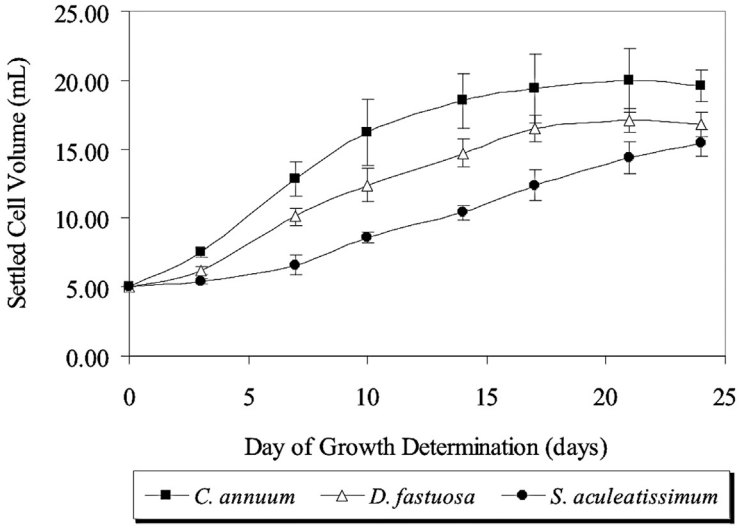
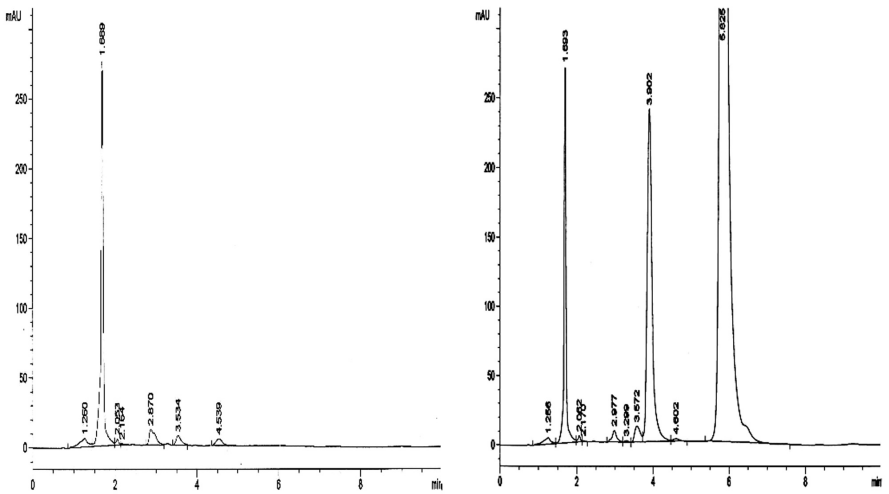


Figure 2. Growth comparison among cell suspension cultures of different plants in appropriate medium for each plant.

Biotransformation of Hydroquinone to Arbutin

HPLC analysis of the cell extract and the liquid medium of the control (untreated cultures) showed no presence of arbutin which indicated that the cultures of these plants did not produce arbutin (Figure 3A). In the hydroquinone-treated samples, HPLC analysis indicated the presence of arbutin (RT = 3.902 minutes; Figure 3B) in both the cells and the liquid media of *C. annuum, D. fastuosa* and *S. aculeatissimum* cell-suspension cultures after 24 hours of hydroquinone addition. For cell-suspension culture of *C. annuum* (Figure 4), the amount of arbutin rapidly increased to a maximum level between day 2 and day 5, after which the arbutin content significantly decreased. Cell-suspension cultures of *C. annuum* showed the

highest amount of arbutin were 292.62±26.49 mg/mL in the medium and 76.09±35.60 mg/mL in the cells on day 2. The amount of arbutin in the media was 2.7 to 4.5 times higher than that in the cells. The production of arbutin by cell-suspension culture of *D. fastuosa* ranged from 2.34±0.57 to 12.34±3.61 mg/mL in the medium and from 2.38±0.62 to 7.02±1.06 mg/mL in the cells, and reached the maximum on day 2 when the level in the medium was 1.6 times of that in the cells (Figure 5). For *S. aculeatissimum* cell-suspension culture, the maximum arbutin production was reached on day 2 after HQ addition (Figure 6) when the level in the medium (104.51±14.98 mg/mL) was more than 7-folds higher than that in the cells (14.07±2.21 mg/mL). The total arbutin production by the *C. annuum* system (368.71±57.46 mg/mL) was 3 and 20 times higher than those by the *S. aculeatissimum* (118.57±13.61 mg/l) and *D. fastuosa* systems (18.22±5.00 mg/l), respectively.



(A) (B)

Figure 3. Representative HPLC-UV chromatogram (280 nm) of liquid culture medium of *C. annuum* cell-suspension culture: (A) untreated control; (B) treated with 15.57 mM hydroquinone for 5 days before harvesting. Arbutin and the non-biotransformed hydroquinone showed RT at 3.902 and 5.825 min, respectively.

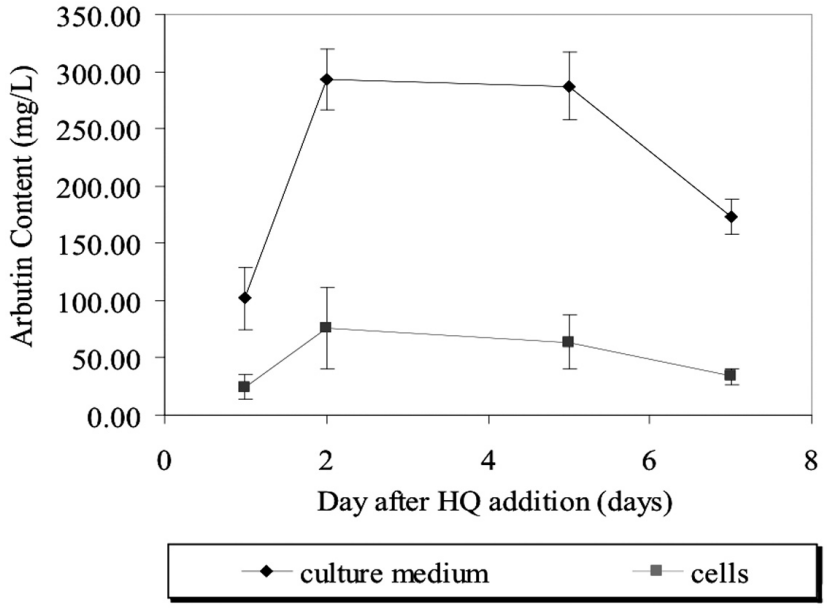


Figure 4. Arbutin content (mg/L) in cells and culture medium of *C. annuum* cell-suspension cultures. The cells and the medium were harvested on day 1, 2, 5 and 7 after the addition of HQ and were analyzed separately (n=3).

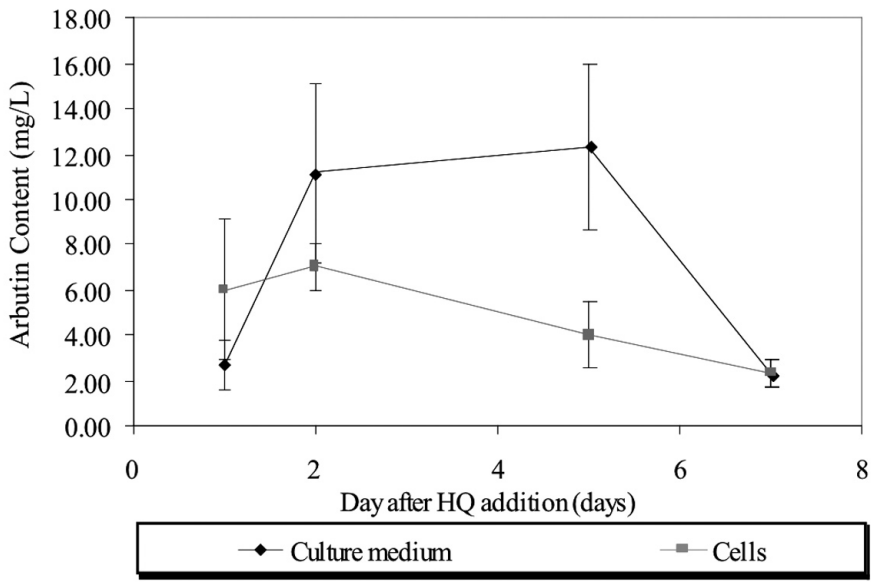


Figure 5. Arbutin content (mg/L) in cells and culture medium of *D. fastuosa* cell-suspension cultures. The cells and the medium were harvested on day 1, 2, 5 and 7 after the addition of HQ and were analyzed separately (n=3).

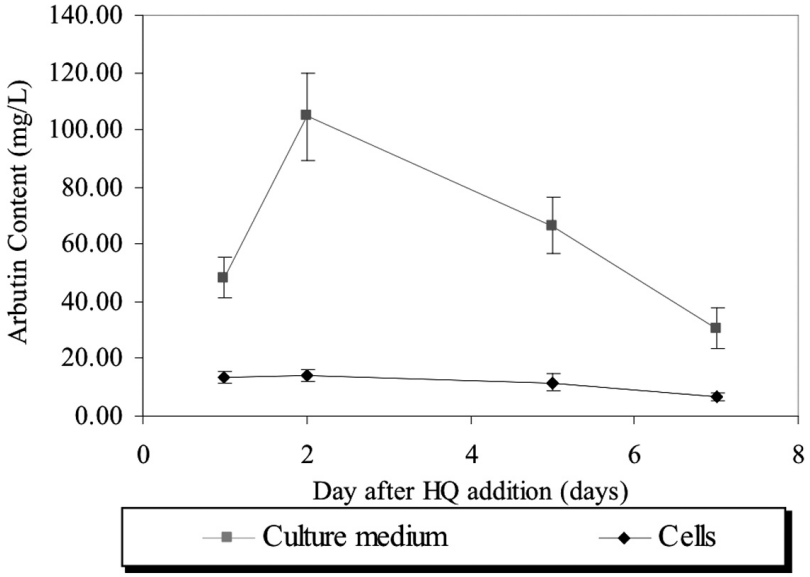


Figure 6. Arbutin content (mg/l) in cells and culture medium of *S. aculeatissimum* cell-suspension cultures. The cells and the medium were harvested on day 1, 2, 5 and 7 after the addition of HQ and were analyzed separately (n=3).

Another interesting point in this study was that in all three plant systems, the amounts of arbutin detected in the culture media were much higher than those in the cells. The result was similar to that reported by Duskova et al., (2005) in which the cell cultures of Schisandra chinensis were able to produce 5.08% of arbutin and released it into the culture media. However, it was in contrast with that reported by Skrzypczak-Pietraszek et al., (2005) in which the cell cultures of the plants studied retained the biotransformed arbutin within the cells. The difference between the two cases could be due to the unique properties of cell cultures of each plant, the morphology of the cells as well as the culture conditions. Two possible hypotheses for this finding are that (1) the exogenously-added HQ molecules were taken up from the media into the cells where they were enzymatically biotransformed to arbutin. The more polar arbutin molecules were then released back into the media in a substantial amount, leaving only a trace amount in the cells or (2) the enzyme was released during the culture period from the cells into the media and the biotransformation took place upon the addition of hydroquinone. To clarify this question, 2-week-old cell-suspension cultures of *C. annuum* were aseptically filtered to separate the cells from the medium. Hydroquinone (15.57 mM) was then added into the cell-free media and maintained on the shaker in the dark condition. The amounts of arbutin in the media analyzed on day 1, 2, 5 and 7 after the addition of HQ were 16.72±1.55, 14.46±2.36, 14.29±0.95 and 15.04±1.26 mg/l, respectively. The much lower amount of arbutin in the cell-free media compared to that in the media of the whole cultures suggested that the enzyme was released into the media in only a limited amount while the majority

of the biotransformation was likely to have taken place in the cells. Further studies are required to fully understand this aspect. We are currently investigating in details the effects of other factors on the production of arbutin (e.g., medium compositions, age of culture, HQ concentration) and developing an optimized arbutin-producing system based on cell-suspension culture of *C. annuum*.

CONCLUSION

Cell-suspension cultures of *C. annuum, D. fastuosa* and *S. aculeatissimum* showed the ability to convert exogenously-supplied hydroquinone into arbutin. The biotransformation likely took place in the cells where a small portion of the produced arbutin was retained. The majority of arbutin was released into the culture media, making these systems were potential candidates for the development of arbutin-producing systems in an industrial scale. While the cell cultures of other *Datura* species have been previously reported for the ability to biotransform hydroquinone to arbutin, this study reports for the first time the ability of cell-suspension cultures derived from the other two genera of the Solanaceae, *Capsicum* and *Solanum*, to carry out such task. The *C. annuum* cell-suspension culture system, in particular, not only produced reasonable amount of arbutin from the added hydroquinone, but also showed fast growth. Therefore, it should be studied in a larger scale as potentially arbutin-producing system.

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REFERENCES

Casas, D.A., S.I. Pitta-Alvarez, and A.M. Giulietti. 1998. Biotransformation of hydroquinone by hairy roots of *Brugmansia candida* and effect of sugars and free-radical scavengers, Appl. Biochem. Biotech. 69: 127-136.

Duskova, J, J. Dusek, and L. Jahodar. 1999. Biotransformation of hydroquinone to arbutin in *in vitro* cultures. Herba Polonica 45: 23-26.

Duskova J, J. Dusek, L. Jahodar, and F. Poustka. 2005. Arbutin and salicin: The possibilities of their biotechnological production. Ceska a Slovenska Farmacie 54: 78-81.

Huang, S.L., Y.L. Zhu , Y.J. Pan, and S.H. Wu. 2004. Synthesis of arbutin by two-step reaction from glucose. J. Zhejiang Univ. Sci. 5: 1509-1511.

Inomata, S., M. Yokoyama, S. Seto, and M. Yanagi. 1991. High-level production of arbutin from hydroquinone in suspension cultures of *Catharanthus roseu*s plant cells. Appl. Micro. Biotech. 36: 315-319.

Jahodar, L., J. Duskova, M. Polasek, and P. Papugova. 1999. Different kinetics of hydroquinone depletion in various medicinal plant tissue cultures producing arbutin. Pharmazie 54(3): 234-235.

Kittipongpatana, N., A. Chaiwan , U. Pusod , and O.S. Kittipongpatana . 2006. High performance liquid chromatographic method for separation and quantitative analysis of arbutin in plant tissue cultures. CMU Journal. in press.

Lutterbach, R., and J. Stoeckigt. 1992. High-yield formation of arbutin from hydroquinone by cell-suspension cultures of *Rauwolfia serpentina*. Helvetica Chimica Acta 75: 2009-2011.

Murashige, T., and F.Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum 15: 473-497.

Skrzypczak-Pietraszek, E., A. Szewczyk, A. Piekoszewska , and H.Ekiert. 2005. Biotransformation of hydroquinone to arbutin in plant *in vitro* cultures - preliminary results. Acta Physiologiae Plantarum 27: 79-87.

Suzuki, T., T. Yoshiaki, M. Tabata , and Y. Fujita. 1987. Potential of *Datura inoxia* cell suspension cultures for glucosylating hydroquinone. Plant Cell Rep. 6: 275-278.

Yokoyama, M. 1996. Industrial application of biotransformations using plant cell cultures. p.79 In: M. Misawa and F. DiCosmo (eds). Plant cell culture secondary metabolism: toward industrial application. Boca Raton: CRC Press.

Zhao, M., J. Ding, J. Liu, and B. Hu. 2001. Studies on arbutin biosynthesis by hairy root of *Panax ginseng* C. A. Mayer. Zhongguo Zhongyao Zazhi 26: 819-822.