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Full Length Research Paper

Effect of precursors on flavonoid production by *Hydrocotyle bonariensis* callus tissues

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Callus tissue of *Hydrocotyle bonariensis* was initiated from the leaf of *H. bonariensis* treated with 2 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg/l kinetin. The culture was kept at 25°C, under light (cool white fluorescent tubes, 1200 lux). To optimize the precursors to increase the production of flavonoid, different precursors were used. The data showed that 4 mg/l proline produced the highest flavonoid yield (10.77 ± 0.25 mg/g DW). The increase in proline concentration did not significantly increase the production of flavonoid. The highest flavonoid yield (10.59 ± 0.18 mg/g DW) was produced in 1 mg/l of glutamine. No significant increase was attained in the flavonoid yield in callus treated with 2, 3 mg/l compared with the control. Phenylalanine at the concentration of 3 mg/l, successfully triggered the production of flavonoid (11.43 ± 0.12 mg/g DW), which was 23% higher than the control. The highest flavonoid production was attained in calluses treated with 4 mg/l of naringenin; and it was 19.72% higher compared with the control.

Key words: Flavonoids, cell culture, amino acid, precursor.

INTRODUCTION

Hydrocotyle bonariensis is widely used for the treatment of tuberculosis, relieving the pain of rheumatism and arthritis, to increase brain capacity and for longevity (Vimala et al., 2003). *Hydrocotyle bonariensis* Comm. ex Lam (Apiacae), locally known as *Pegaga Embun,* is a perennial prostrate herb and found mostly in tropical and subtropical region of the world (Reed, 2007). Flavonoids are a large group of polyphenolic compounds and remarkable plant metabolites that occur commonly in plants (Nikolova and Gevrenova, 2006). Flavonoid-derived natural products evolved in parallel with plants and their role in every day plant life is to protect them from environmental biotic and abiotic stresses. It is long known that these natural gifts are also beneficial for human health, either as direct medicines or indirectly as nutritional supplements (Ververidis et al., 2007). It has been reported that, the leaves of this plant contain alkaloids, flavonoids, tannins, phenolic compounds and saponins as bioactive components (Ajani et al., 2009). Plant flavonoids are important in the diet because of their beneficial effects on human health (Gebhardt et al., 2005). Cell-culture-derived flavonoids can be more easily separated in an intact polymeric form than flavonoids within complex plant tissues and overall the concentrations can be significantly enhanced in callus cultures through micro environmental control and precursor feeding to provoke metabolite production (Lila et al., 2005). In order to enhance the synthesis of secondary metabolites, several organic compounds can be added to the culture medium (Namdeo et al., 2007). The concept is based on the idea that any compound, which is an intermediate in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product (Rao and Ravishankar, 2002). The regulation of amino acids pathway under some stress condition dominated by the need for secondary metabolite derived from the pathway (Zia et al., 2007). Isoflavones and flavonoids originated from phenylalanine, an upstream metabolic precursor through phenylpropanoid pathway. Supplementation of phenylalanine is expected to increase elevated level of target compound (Shinde et al., 2009). In this work callus of *H. bonariensis* was established and grown in DKW (Driver and Kuniyuki, 1998) medium. The main objective of this study was to optimize precursor concentration to

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obtain the optimum callus growth and flavonoids production of *H. bonariensis*.

MATERIALS AND METHODS

Plant materials

Hydrocotyle bonariensis was collected from greenhouse at University Putra Malaysia and surface disinfected using 70%(v/v) of ethanol for 1 min, followed by 15% aqueous sodium hypochlorite solution for 20 min, then rinsed three times in sterile distilled water. Leaves of *H. bonariensis* were cut into small pieces (0.5 cm × 0.5 cm) and aseptically placed on MS medium (Murashige and Skoog, 1962) supplemented with B5 vitamins, gelrite (2.75% w/v), 3% sucrose and supplemented with 2 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg/l kinetin (Figure 9). The pH of the medium was adjusted to 5.7 with NaOH or HCl before autoclaving at 121°C for 15 min. The explants were incubated under 16 h photoperiod of 1200 lux at 25 ± 2°C until callus was induced. The callus tissues formed from the leaf explants were subcultured onto fresh medium every three weeks.

Effect of phenylalanine

A stock solution of 1 mg/ml (w/v) of concentration was prepared by dissolving 10 mg of phenylalanine separately with 10 ml of water. Phenylalanine studied was microfilter sterilized using 0.2 µm of polyethersulfone membrane (Whatman, UK) because it is heat sensitive. Hence, autoclave process is inappropriate.

Effect of proline

Proline stock solution of 1 mg/ml (w/v) of concentration was prepared by dissolving 10 mg of precursor separately with 10 ml of distilled water and microfilter sterilized using 0.2 µm of polyethersulfone membrane (Whatman, UK).

Effect of glutamine

Glutamine stock solution of 1 mg/ml (w/v) of concentration was prepared by dissolving 10 mg of glutamine separately with 10 ml of distilled water and microfilter sterilized using 0.2 µm of polyethersulfone membrane (Whatman, UK).

Effect of naringenin

For naringenin, a stock solution was dissolved in ethanol and subsequent dilution up to 100 times was made up with distilled water. Microfilter sterilized using 0.2 µm of polyethersulfone membrane (Whatman, UK) because naringenin is heat sensitive. Hence, autoclave process is inappropriate. The corresponding concentrations of naringenin were then added directly to the autoclaved culture media.

Extraction and determination of flavonoids

Extraction of flavonoids from the dried callus, were performed as described by Jia et al. (1999). Quantitative determination of flavonoids was achieved with $Al(NO_3)_3$ reagent as described by Zhang et al. (1992) and Liu et al. (2002). The absorbance was read at 510 nm using quercetin as the standard. Each treatment was replicated three times.

Statistical analysis

The experiments were independently repeated three times under the same conditions and the concentrations and all analyses were performed in triplicates. Results are expressed as the g FW (fresh weight) for biomass and mg DW (dry weight) for flavonoid accumulation in treated *in vitro* cultures compared with untreated samples. The Graphs show the flavonoid accumulation using Microsoft® Excel. Error bars of graphs show the standard error of mean value (±S.E.M.). The data were analyzed using one-way ANOVA followed by Duncan's multiple range test for mean comparison at $P = 0.05$.

RESULTS AND DISCUSSION

Influence of phenylalanine on biomass and flavonoid production

Figure 1 demonstrated the effects of phenylalanine on callus growth in *H. bonariensis*. Data showed that, the supplementation of phenylalanine at the range of 2 to 5 mg/l did not significantly ($p > 0.05$) cause variation on the callus biomass. Phenylalanine (2 mg/l) produced maximum biomass (3.14 ± 0.11 g FW/culture) followed by 3, 4, 5 mg/l and control. The lowest growth was observed in 1 mg/l phenylalanine (2.3 ± 0.09 g FW/culture). Phenylalanine enhanced the biomass accumulation by 12.71% with supplementation of 2 mg/l.

Flavonoid production by phenylalanine is shown in Figure 2. The data revealed that, the most suitable concentration for the highest flavonoid production was 3 mg/l, with flavonoid production of 11.43 ± 0.12 mg/g DW, which was 23% higher than the control. Higher concentration of flavonoid seemed to be unsuitable for flavonoid production and the minimum flavonoid accumulation was obtained in 5 mg/l phenylalanine (9.06 ± 0.13 mg/g DW).

The optimized concentration of phenylalanine for paclitaxel (Taxol) production from *Taxus chinensis* was 15 mg/l (Luo and He, 2004) and this concentration is 5 times higher than optimum concentration for flavonoid production. Khosroushahi et al. (2006) reported that, in *Taxus baccata,* addition of phenylalanine increased the Taxol amount. The effect of precursor feeding (phenylalanine) on the production of isoflavones in *Psoralea corylifolia* hairy root culture demonstrated that, phenylalanine at 2 mM concentration increased the production of daidzein and genistein by 1.3 fold compared with the control. Daidzein and genistein levels were greatly inhibited when concentration of phenylalanine was increased to 10 mM (Shinde et al., 2009). Artemisinin was detected in *Artemisia absinthium* callus culture by adding 12.5 mg/l phenylalanine to the

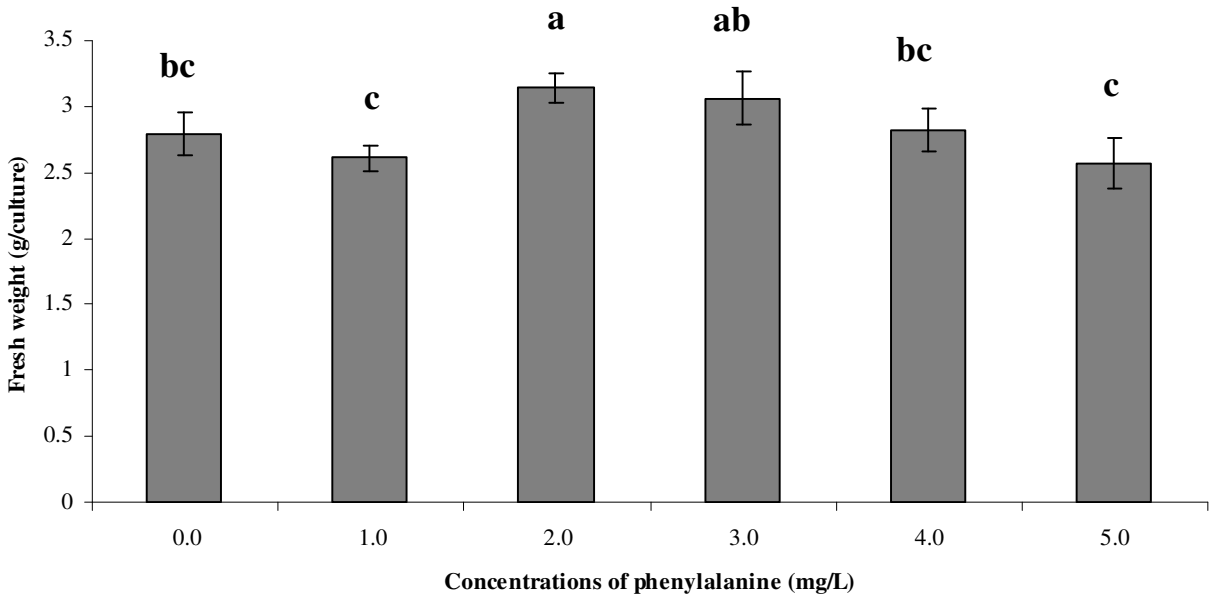

Figure 1. Effects of the phenylalanine on the fresh weight of *H. bonariensis* callus culture in basal DKW medium supplemented with 2,4D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are mean ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

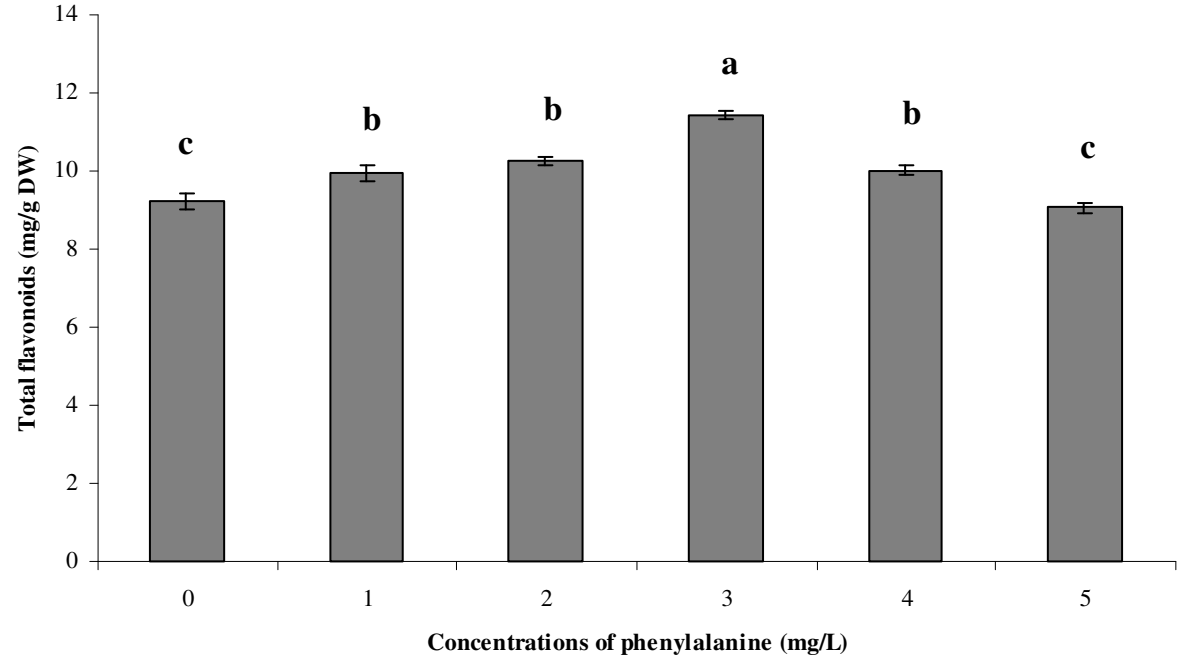

Figure 2. Total flavonoids accumulation in *H. bonariensis* callus culture on phenylalanine in basal DKW medium supplemented with 2,4-D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are means ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

medium (Zia et al., 2007). Phenylalanine at low concentration (< 33 mg/l) showed a negative effect on the cell growth and a significant positive ($p < 0.05$) effect on phenylethanoid glycosides biosynthesis. After 20 days culture, the cell biomass and phenylethanoid glycosides content decreased with the increase of phenylalanine

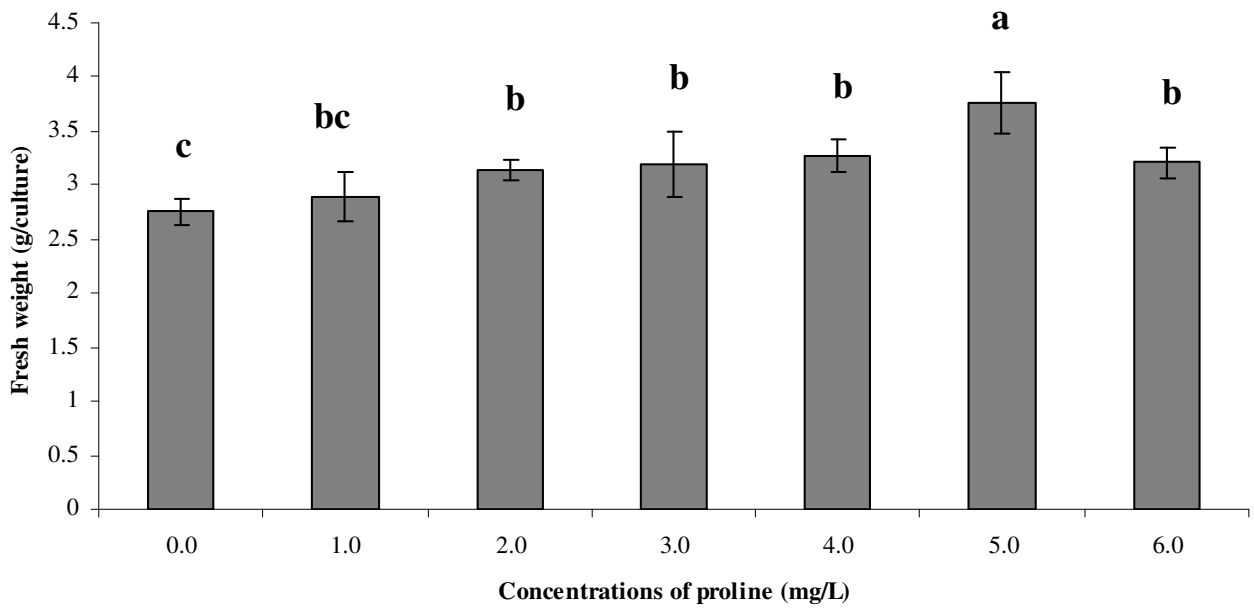

Figure 3. Effects of the proline on the fresh weight of *H. bonariensis* callus culture in basal DKW medium supplemented with 2,4D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are mean ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

concentration when the precursor concentration was over 33 mg/l (Ouyang et al., 2005). Flavonoid originated from phenylalanine, an upstream metabolic precursor through phenylpropanoid pathway. Considering this phenylalanine supplementation which is expected to increase the metabolic flux through phenyl-propanoid biosynthetic pathway and elevate the level of targeted compound. Phenylalanine supplementation has been reported to enhance secondary metabolite production in plant cell cultures (Shinde et al., 2009).

Effects of proline on biomass and flavonoid production

Figure 3 demonstrated the effect of proline on callus growth. Maximum growth was observed at 5 mg/l proline (3.76 ± 0.29 g FW/culture and 0.088 g DW/culture). With increasing proline concentration, cell growth was enhanced. All treatment containing proline produced growth higher than the control. Proline compared with glutamine and phenylalanine was used in higher concentration. However, increasing growth was not detected in callus with concentrations higher than 5 mg/l. In terms of flavonoid production by proline (Figure 4), the data showed that 4 mg/l proline produced the highest flavonoid yield (10.77 ± 0.25 mg/g DW). Increasing the proline concentration did not significantly increase ($p > 0.05$) the flavonoid production. The result demonstrated that, concentration higher than 4 mg/l proline inhibited the flavonoid production. Based on the data obtained, there was no significant difference ($p > 0.05$) between the control and proline (1 and 2 mg/l), and also no increase was detected in flavonoid treated with 5 to 6 mg/l proline. Alkaloid accumulation by *Catharanthus roseus* was enhanced by the addition of amino acid L-tryptophan, however, it did not affect the culture growth (Whitmer et al., 2002). This result is in agreement with the effect of proline in flavonoid production. In addition, proline was able to increase the biomass in *H. bonariensis.* In contrast, the supplementation of L-valine or L-leucine at the final concentrations of 0 to 5 mM, which had no significant stimulating effects ($p > 0.05$) on the production of adhyperforin or hyperforin in *Hypericum perforatum* shoot cultures. This implies that, although we showed that L-valine is a precursor for hyperforin, the concentration of valine seems not to be the limiting factor in the biosynthesis of hyperforin in *H. perforatum* shoot cultures (Karppinen et al., 2007). Addition of cystine and leucine (12.5 mg/l each) in the medium of *A. absinthium* callus resulted in the production of 2.8 and 0.58 µg/g DW artemisinin, respectively. However, artemisinin was found in callus cultured on MS medium containing 12.5 mg/l of valine (Zia et al., 2007). Low concentrations (<18 mg/l) of L-tyrosine had positive effects on both cell growth and phenylethanoid glycosides biosynthesis. In all the concentrations tested, tyrosine at 36 mg/l gave the highest biomass, at 0.1 mmol/l gave the highest phenylethanoid glycosides production (Ouyang et al., 2005).

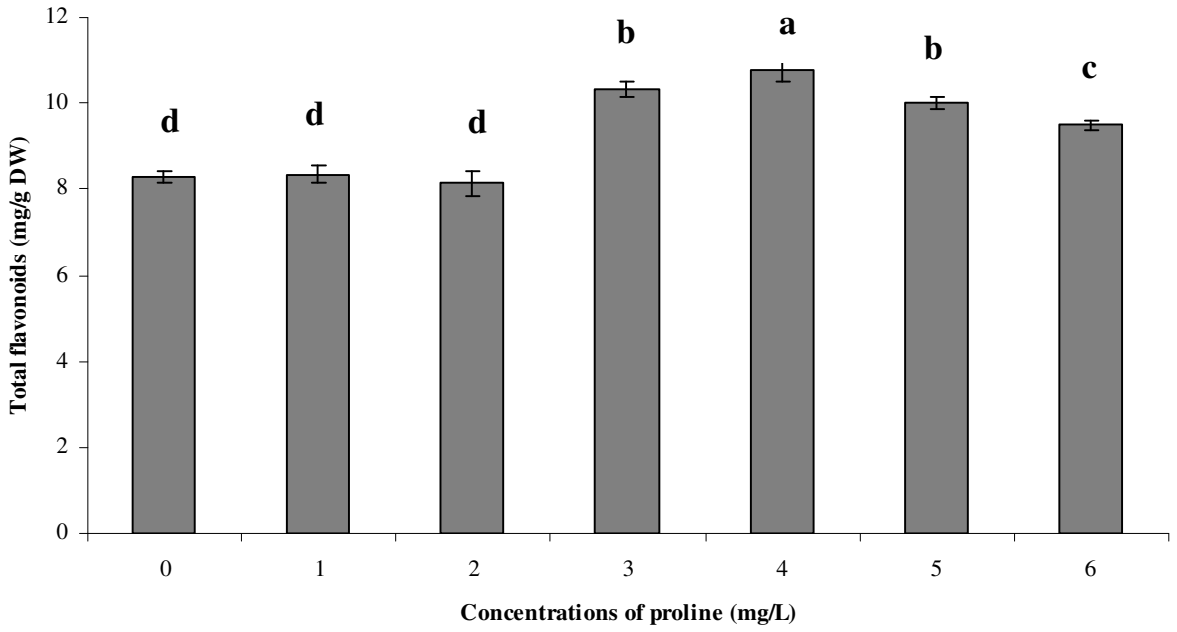

Figure 4. Total flavonoids accumulation in *H. bonariensis* callus culture on proline in basal DKW medium supplemented with 2,4-D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are means ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

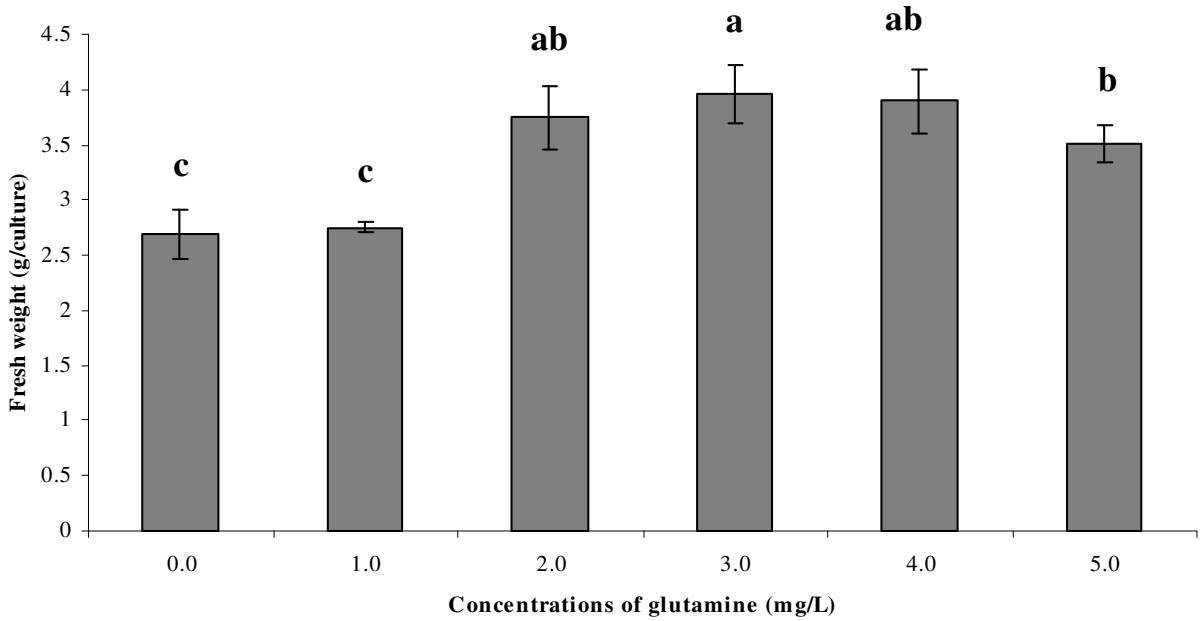

Figure 5. Effects of the glutamine on the fresh weight of *H. bonariensis* callus culture in basal DKW medium supplemented with 2,4D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are mean ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

Effect of glutamine on cell growth and flavonoid accumulation

The effects of glutamine on callus growth are shown in Figure 5. Cells treated with 3 mg/l of glutamine showed the highest biomass yield (3.95 ± 0.25 g FW/culture and 0.083 g DW/culture). Glutamine (1 mg/l) produced the lowest cell growth and there was no significant difference

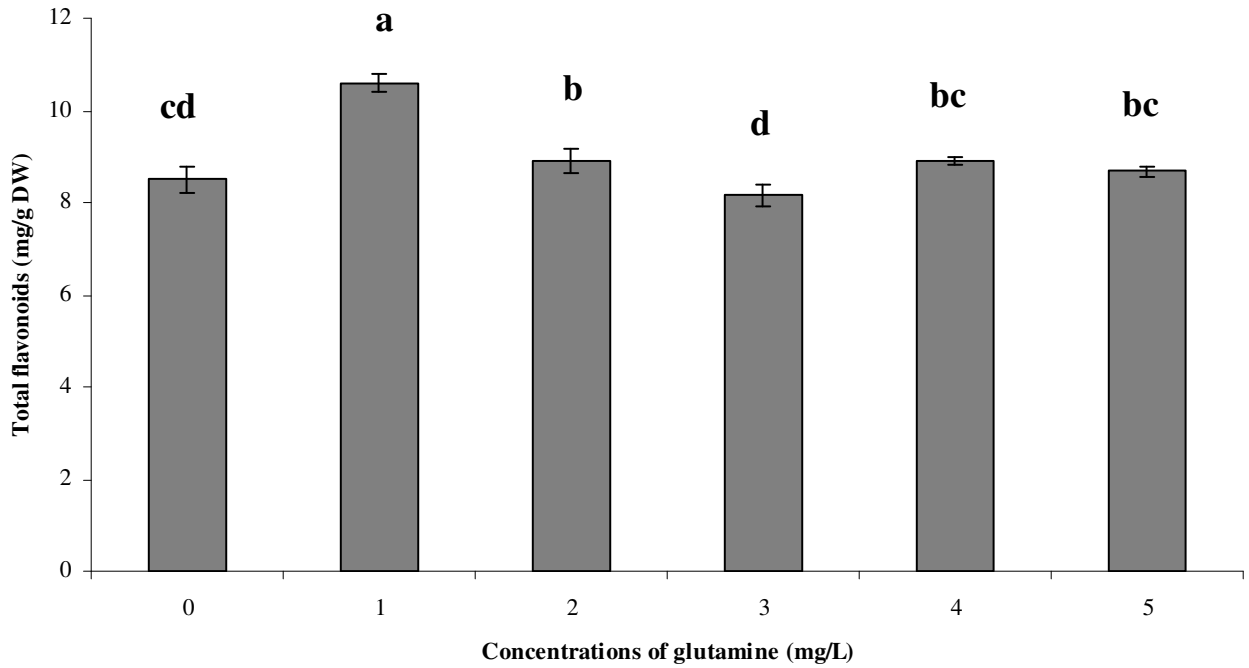

Figure 6. Total flavonoids accumulation in *H. bonariensis* callus culture on glutamine in basal DKW medium supplemented with 2,4-D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are means ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

($p > 0.05$) compared with control. The data revealed that, an increase in glutamine concentration resulted to an increase in cell growth.

Flavonoid yield was not significantly altered ($p > 0.05$) by applied glutamine except 1 mg/l glutamine (Figure 6). The highest flavonoid yield (10.59 ± 0.18 mg/g DW) was produced in 1 mg/l of glutamine. No significant increase ($p > 0.05$) was attained in the flavonoid yield in callus treated with 2 and 3 mg/l compared with the control. The concentrations higher than 1 mg/l, seem to be suitable in flavonoid production except all these concentrations increase the biomass growth to considerable level.

Treatment with L-threonine, as precursor, stimulated the production of adhyperforin in *H. perforatum* shoot cultures. The adhyperforin concentration (0.68 ± 0.11 mg/g DW) was significantly higher ($p < 0.05$) than that of the control cultures (0.35 ± 0.04 mg/ g DW) when 3 mM of L-threonine was supplied. Concentrations higher than 3 mM were not equally effective in the stimulation of adhyperforin production. The high concentrations of L-threonine could have caused inhibition in the adhyperforin biosynthetic pathway leading to inefficient biotransformation. The hyperforin concentration in shoot cultures of *H. perforatum* was not significantly affected ($p > 0.05$) by treatment with L-threonine (Karppinen et al., 2007).

Callus of *A. absinthium* showed the presence of artemisinin by the addition of 12.5 mg/l glutamine as precursor (Zia et al., 2007). Coniferyl alcohol as a precursor used in suspension culture of *S. marianum* showed the changes in silymarin complex production. Silydianin was detected mainly in the control samples of cultivated cells. A significant increase ($p < 0.05$) of silydianin was observed only after 72 h of the application of 46 µM coniferyl alcohol. But a significant increase ($p < 0.05$) accumulation of taxifolin (flavanole) in nutrient medium was observed after 72 h of treatment with 92 µM of coniferyl alcohol (Tumova et al., 2006).

Influence of the naringenin on the biomass and flavonoid accumulation

Precursor feeding at appropriate concentrations can promote the accumulation of secondary metabolites (Ouyang et al., 2005). Naringenin is one of the flavonoids produced in the beginning of the flavonoid pathway, so it can be used as other flavonoid precursors. Figure 7 exhibited the effects of naringenin supplementation and their controls. None of the concentrations tested managed to achieve cell growth higher than the control. At 4 mg/l, the biomass was 2.52 ± 0.06 g FW/culture and0.070 g DW/culture, which was 6.62% lower than the control. Comparable observation was also detected in callus culture of *Rhodiola rosea* treated with cinnamyl alcohol; the feeding precursor did not increase the cell biomass more than the control (György et al., 2004). Figure 8 demonstrated the effect of naringenin on

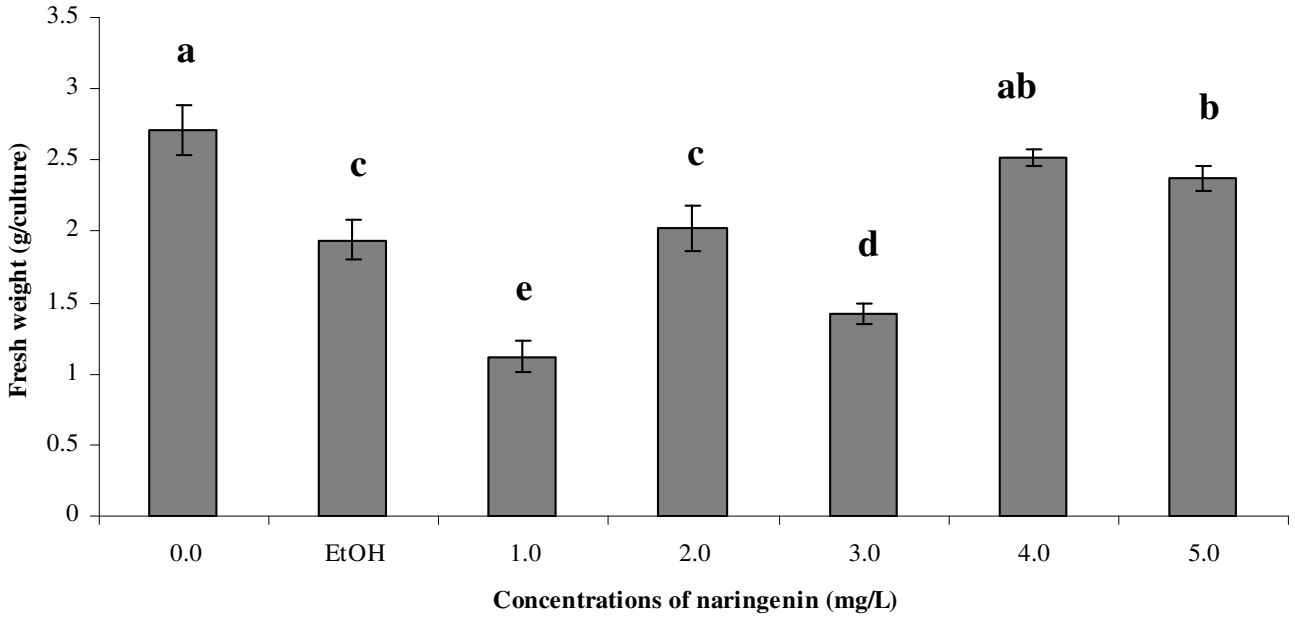

Figure 7. Effects of the naringenin on the fresh weight of *H .bonariensis* callus culture in basal DKW medium supplemented with 2,4D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are mean ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

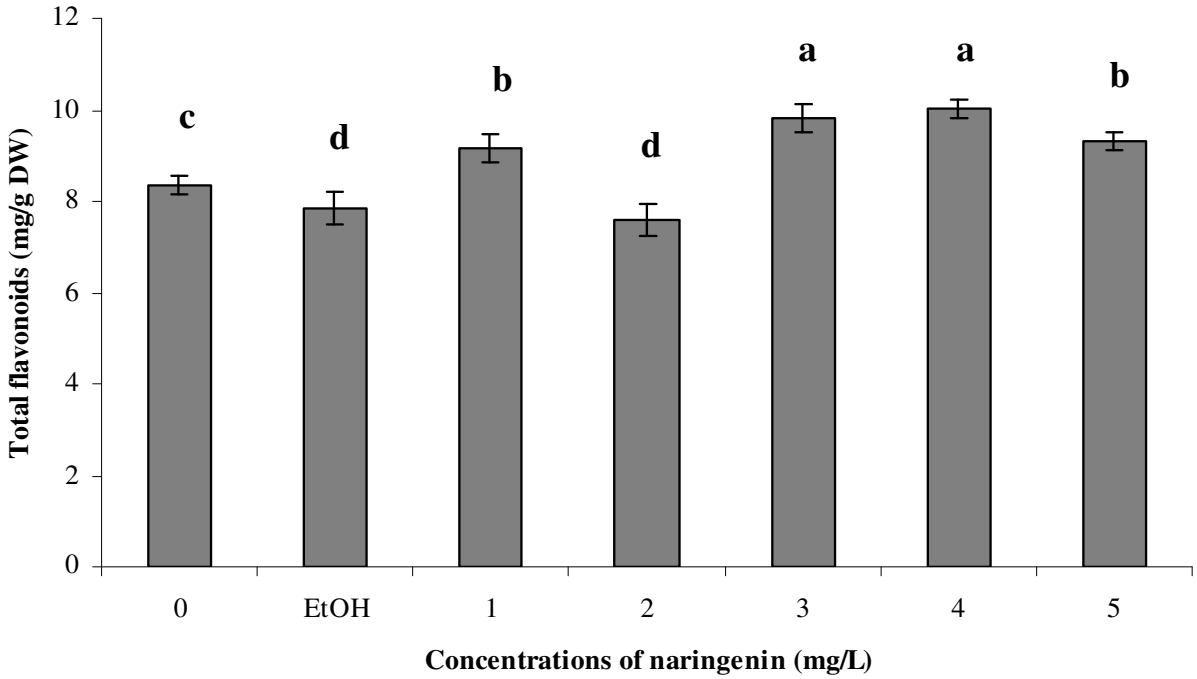

Figure 8. Total flavonoids accumulation in *H. bonariensis* callus culture on naringenin in basal DKW medium supplemented with 2,4-D: kinetin (2:1mg/l) incubated at 25 ± 2°C. Values are means ± S.E.M. of three experiments. Means with the same letter are not significantly different by Duncan's multiple range test (P = 0.05).

flavonoid production in callus cultures of *H. bonariensis*. The highest flavonoid production was attained in calluses treated with 4 mg/l of naringenin. The result shows no significant difference ($p > 0.05$) in the content of flavonoids

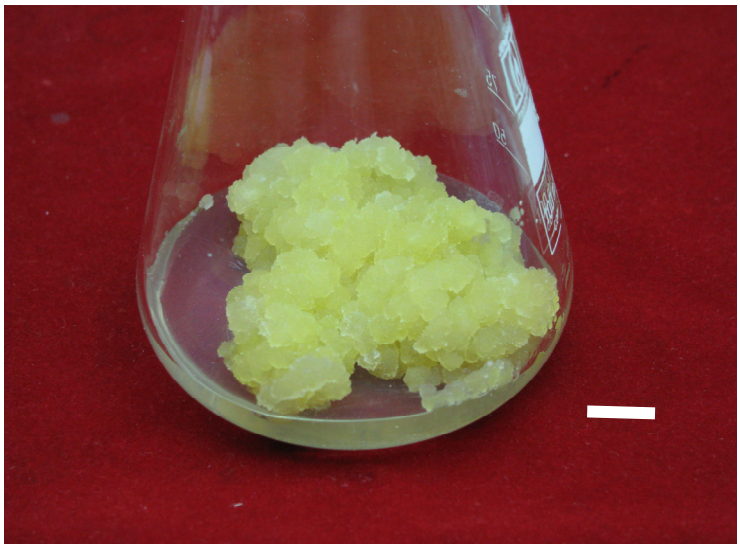

Figure 9. Callus culture of *H. bonariensis* on DKW medium supplemented with 2 mg/l 2,4-D and 1 mg/l kinetin incubated at 25 ± 2°C. Bar 1 cm.

with the application of 3 and 4 mg/l naringenin, but 4 mg/l naringenin induced the highest amount of flavonoid. Moreover, the yield of flavonoid was 19.72% higher compared with the control. At 2 mg/l, the content of flavonoid was the lowest among the treated cell by naringenin (9.21%). Higher concentration of naringenin (5 mg/l) supplementation was found to decrease flavonoid production.

Stimulation of secondary metabolites by precursor feeding was also observed in *Ipomoea batatas* cell suspension. In this culture, cell growth was decreased, but acylated anthocyanins increased and non-acylated anthocyanins decreased with increasing precursor *p*-coumaric (0 to 2 mM). In addition, feeding of the hydroxyl-cinnamic acid to those cultures resulted in increased accumulation of acylated anthocyanins, both mono and di-acylated (Konczaka et al., 2005). Excess precursors may cause feedback inhibition to the metabolic pathway. It is very important therefore to determine the appropriate precursor concentration in the precursor-feeding test (Ouyang et al., 2005). It was reported that, addition of mevalonates to the culture medium did not alter shikonin production by cultured *Lithospermum erythrorhizon* cells, whereas the addition of p-hydroxybenzoic acid strongly increased shikonin formation. So, the content of p-hydroxybenzoic acid was much higher in shikonin-producing cells than in shikonin-free cells and exogenous addition of p-hydroxybenzoate increased shikonin production (Bulgakov et al., 2001). The effect of naringenin on flavonoid production in *H. bonariensis* was comparable to that of other precursors such as loganin or secologanin, which showed improvement on alkaloid accumulation by *C. roseus* (Whitmer et al., 2002).

REFERENCES

Ajani EO, Salako AA, Sharlie PD, Akinleye WA, Adeoye AO, Salau BA, Adebawo OO (2009). Chemopreventive and remediation effect of *Hydrocotyl bonariensis* Comm. Ex Lam (Apiaceae) leave extract in galactose induced cataract. J. Ethnopharmacol. 123(1):134-142.

Bulgakov VP, Kozyrenko MM, Fedoreyev SA, Mischenko NP, Denisenko VA, Zvereva LV, Pokushalova TV, Zhuravlev YN (2001). Shikonin production by p-fluorophenylalanine resistant cells of *Lithospermum erythrorhizon*. Fitoterapia 72:394-401.

Driver JA, Kuniyuki AH (1998). Biochemical plant cell and tissue culture. Duchefa Biochemic BW: Haarlem, The Netherlands.

Gebhardt Y, Witte S, Forkmann G, Lukacin R, Matern U, Martens S (2005). Molecular evolution of flavonoid dioxygenases in the family Apiaceae. Phytochemistry, 66:1273-1284.

György Z, Tolonen A, Pakonen M, Neubauer P, Hohtola A (2004). Enhancing the production of cinnamyl glycosides in compact callus aggregate cultures of *Rhodiola rosea* by biotransformation of cinnamyl alcohol. Plant Sci. 166:229-236.

Jia ZS, Tang MM, Wu JM (1999). The determination of flavonoids contents in mulberry and their scavenging effect on superoxide radicals. Food Chem. 64:555-559.

Karppinen K, Hokkanen J, Tolonen A, Mattila S, Hohtola A (2007). Biosynthesis of hyperforin and adhyperforin from amino acid precursors in shoot cultures of *Hypericum perforatum*. Phytochemistry, 68:1038-1045.

Khosroushahi AY, Valizadeh M, Ghasempour A, Khosrowshahli M, Naghdibadi H, Dadpour MR, Omidi Y (2006). Improved taxol production by combination of inducing factors in suspension cell culture of *Taxus baccata*. Cell Biol. Int. 30:262-269.

Konczaka I, Teraharab N, Yoshimotoc M, Nakatanid M, Yoshinagac M, Yamakawa O (2005). Regulating the composition of anthocyanins and phenolic acids in a sweetpotato cell culture towards production of polyphenolic complex with enhanced physiological activity Trends Food Sci. Technol. 16:377-388.

Lila MA, Yousef GG, Jiang Y, Weaver CM (2005). Flavonoid mixtures from cell cultures. J. Nutr. 135: 1231-1235.

Liu M, Li Q, Weber C, Li CY, Brown J, Liu RH (2002). Antioxidant and antiproliferative activities of raspberries. J. Agric. Food. Chem. 20: 2926-2930.
Luo J, He GY (2004). Optimization of elicitors and precursors for paclitaxel production in cell suspension culture of *Taxus chinensis* in the presence of nutrient feeding. Process Biochem. 39:1073-1079.
Namdeo AG, Jadhav TS, Rai PK, Gavali S, Mahadik KR (2007). Precursor feeding for enhanced production of secondary metabolites. Pharmacogn. Rev. 1(2): 227-231.
Nikolova M, Gevrenova R (2006). A HPLC analysis on interpopulational variations in the flavonoid composition of *Veronica chamaedrys*. Int. J. Bot. 2(1): 7-10.
Ouyang J, Wang XD, Zhao B, Wang YC (2005). Enhanced production of phenylethanoid glycosides by precursor feeding to cell culture of Cistanche deserticola. Process Biochem. 40:3480-3484.
Rao SR, Ravishankar GA (2002). Plant cell cultures: Chemical factories of secondary metabolites. Biotechnol. Adv. 20:101-153.
Reed DW (2007). Wildflowers of the Southeastern United States. www.2bnTheWild.com. Accessed on 5 April 2007.
Shinde AN, Malpathak N, Fulzele DP (2009). Enhanced production of phytoestrogenic isoflavones from hairy root cultures of *Psoralea corylifolia* L. using elicitation and precursor feeding. Biotechnol. Bioprocess Eng. 14: 288-294.
Tumova L, Rimakova J, Tuma J, Dusek J (2006). *Silybum marianum in vitro*-flavonolignan production. Plant Soil Environ. 52(10):454-458.
Ververidis F, Trantas E, Douglas C, Vollmer G, Kretzschmar G, Panopoulos N (2007). Biotechnology of flavonoids and other phenylpropanoid-derived natural products. Part II: Reconstruction of multienzyme pathways in plants and microbes. Biotechnol. J. 2:1235-1249.
Vimala S, Adenan MI, Ahmad AR, Shahdan R (2003). Nature's choice to wellness: Antioxidant vegetables/ulam. Forest Research Institute Malaysia: Selangor.
Whitmer S, Heijden Rvd, Verpoorte R (2002). Effect of precursor feeding on alkaloid accumulation by a tryptophan decarboxylase over-expressing transgenic cell line T22 of *Catharanthus roseus*. J. Biotechnol. 96:193-203.
Zhang YL, Zhu M, Zhao BH (1992). The effect of Armillaria mellea elicitor on alkaloid production in tissue cultures of *Corydalis yanhusuo* Acta. Bot. Sin. 34:658-661.
Zia M, Mannan A, Chaudhary MF (2007). Effect of growth regulators and amino acids on artemisinin production in the callus of *Artemisia absinthium*. Pak. J. Bot. 39(2):799-508.