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Effects of Precursor Supplementation on the Production of Triterpenes by *Centella asiatica* Callus Cultures

[1]Anna Ling Pick Kiong, [2]Maziah Mahmood, [3]Nor'aini Mohd Fadzillah and [3]Siti Khalijah Daud
[1]Department of Bioscience, Faculty of Engineering and Science,
Universiti Tunku Abdul Rahman, 53300 Setapak, Kuala Lumpur, Malaysia
[2]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Science,
[3]Department of Biology, Faculty of Science,
Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Malaysia

Abstract: Production of the four targeted triterpenes, asiatic acid, madecassic acid, asiaticoside and madecassoside in leaf derived callus and cell suspension cultures of *Centella asiatica* was compared with its production in whole plant from field and *in vitro* shoot cultures. In callus a cultures, production was found the highest during the third week of culture and the contents declined gradually. The glycosides, madecassoside and asiaticoside contents were found higher than the asiatic acid and madecassic acid in callus cultures. Four triterpenes precursors, squalene, Farnesyl Pyrophosphate (FPP), Isopentenyl Pyrophosphate (IPP) and leucine, which are involved directly or indirectly in the triterpene biosynthetic pathway, were used to increase the triterpenes production. Squalene was found the best precursor in promoting triterpenes production in callus cultures. Squalene treated callus also produced the highest biomass production compared to the other precursors tested.

Key words: *Centella asiatica*, precursor, triterpenes, secondary metabolites, *in vitro* cultures

INTRODUCTION

Centella asiatica (L.) Urban which is an ethnomedical plant used in different continents by diverse ancient cultures and tribal groups. Different uses are claimed for the plant with the most common one being the wound healing agent and constituent of brain tonics for the mentally retarded[1]. These properties have been ascribed to the active principles that belong to the β-amyrin ursolic acid group - the asiatic acid, asiaticoside, madecassic acid and madecassoside. These are pentacyclic triterpenes, found to display chronic venous insufficiency[1], varicose vein and wound healing properties. In the year 1990 alone the estimated annual requirement of *C. asiatica* was around 12,700 t of dry biomass valued at Rs 1.5 billion[2]. The large-scale and unrestricted exploitation of this natural resource to meet its increasing demand by the pharmaceutical industry has caused the depletion of this plant and now it is listed as threatened species by the International Union for Conservation of Nature and National Resources (IUCN) and an endangered species in India[3,4]. Further, there is some evidence of genotypic difference in glycoside content and medicinal properties in *C. asiatica*[5]. Thus, the use of plant cell culture process has been looked at as a potential alternative for the more efficient production of a group of bioactive ingredients of *C. asiatica*.

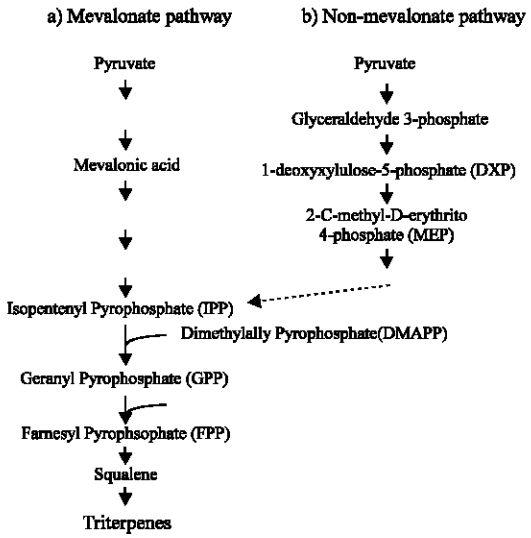

Fig. 1: Pathway of terpenes biosynthesis (a) Mevalonate pathway (b) Non-mevalonate pathway[7-10]

Corresponding Author: Dr. Anna Ling Pick Kiong, Department of Bioscience, Faculty of Engineering and Science, Universiti Tunku Abdul Rahman, 53300 Setapak, Kuala Lumpur, Malaysia
Tel: 603 41079802/132 Fax: 603 41079803

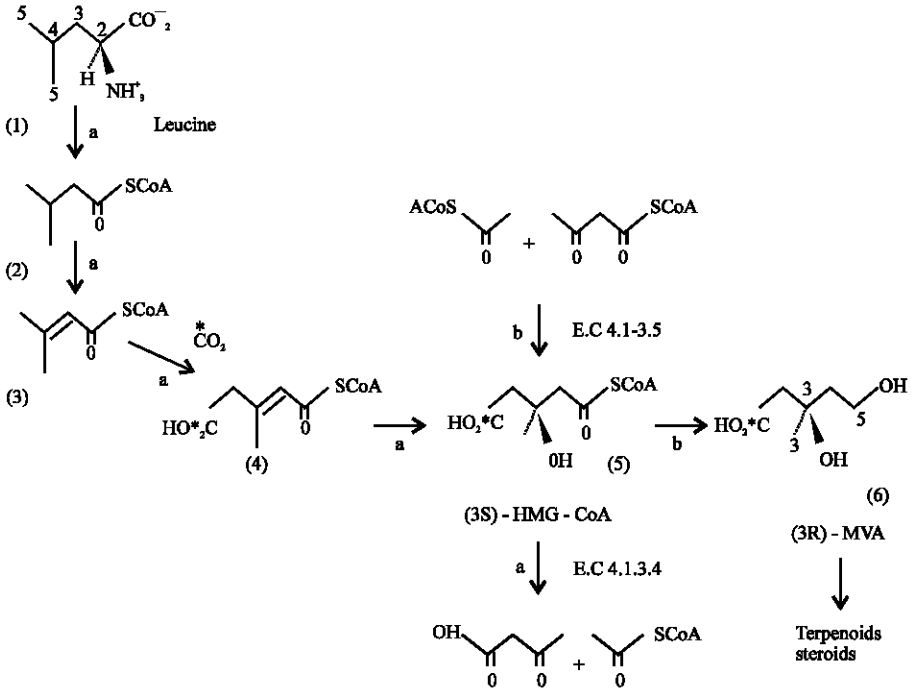

Fig. 2: Cross-over of the catabolic pathway for leucine and the anabolic pathway for terpenoids at HMG-CoA. (1) Leucine (2) Isovaleryl-CoA (3) 3-methylcrotonyl-CoA (4) 3-methylglutaconyl-CoA (5) HMG-CoA (6) Mevalonic acid (MVA). (a) HMG-CoA lyase (E.C 4.1.3.4) (b) HMG-CoA synthase (E.C 4.1.3.5)[11,12]

One of the strategies in enhancing the natural product accumulation is feeding plant cell cultures with commercially available or easily extractable metabolic precursors. Precursor feeding had been an obvious and popular approach to increase secondary metabolite production in plant cell cultures. The concept is based upon the idea that any compound, which is an intermediate in or at the beginning of a secondary biosynthetic route, stands a good chance of increasing the yield of the final product[6]. Precursor feeding is most likely to be adopted in inducing high levels of secondary product formation in intrinsically low-producing cultures. Addition of known or putative precursors in a pathway may enhance the production of secondary metabolites in plant cell cultures if the endogenous level of these precursors is a limiting factor[7]. Feeding metabolic precursors in some instances can also help to identify steps in a pathway at which the activity of an enzyme or the availability of its substrate might limit the overall flux through the pathway[7].

Precursors of biosynthetic pathways have been used in various plant cell suspension cultures to increase the yield of secondary metabolites. With the aim of increasing the biomass and triterpenes yields in callus cultures of *C. asiatica*, the effects of different triterpenes precursors namely, squalene, Isopentenyl Pyrophosphate (IPP), Farnesyl Pyrophosphate (FPP) and leucine were investigated in this study. Referring to the triterpenes biosynthesis pathway (Fig. 1), squalene is the immediate precursor while IPP and FPP act as the intermediate in the pathway[7-10]. Leucine that involved in the formation of HMG-CoA is considered as the early precursor for the biosynthesis of triterpenes (Fig. 2)[11,12].

MATERIALS AND METHODS

Plant materials: The *C. asiatica* plants used in this study were obtained from Malaysian Agriculture Research and Development Institute (MARDI), Serdang, Selangor between January to June 2003. The mature intact plants (four weeks old) were used in the studies for the determination of triterpenes content. To obtain clean samples, freshly collected samples were rinsed with slow running tap water and then separated into its distinctive parts, namely leaf, petiole, stolon and root.

Initiation of *in vitro* cultures and culture conditions: *In vitro* shoot cultures used for the comparison studies were initiated from the sterile meristem shoot explants of *C. asiatica*. The plantlets were cultured in the basal MS

medium containing 3.0% (w/v) sucrose, 0.5 mg L^{-1} IAA at pH 5.7 and solidified with 0.25% (w/v) gelrite. The cultures were incubated at 25±2°C with a photoperiod of 16 h of fluorescent light. Four weeks old plantlets were extracted and triterpenes content was analyzed.

For the initiation of callus culture, the fresh young leaves collected were washed under running water for 30 min followed by immersion in 20% (v/v) commercial Clorox with three drops of Tween 20 for 15 min under vacuum conditions. The sterile explants were then rinsed three times with sterile distilled water at 5, 10 and 15 min, respectively. Sterile explants with the size of 5x5 mm (leaf) were aseptically cultured into a basal Murashige and Skoog (MS) medium supplemented with 2.0 mg L^{-1} (w/v) 2,4-D and 1.0 mg L^{-1} (w/v) kinetin, B5 vitamins, 3% (w/v) sucrose and 0.25% (w/v) gelrite at pH 5.7. Callus obtained was carefully separated from the explant source and transferred into the same fresh medium. After several subcultures, the callus was used as the inoculums for the treatments. In these studies, all the treatments were carried out in the transparent glass tubes (8.4x2.4 cm). A 10 mL of the medium was placed in the tubes and covered with a layer of heavy-duty aluminum foil. The autoclaved media were left to stand (45°) until the media is fully solidified. The cultures were incubated at 25±2°C with a photoperiod of 16 h fluorescent light. The Dry Weight (DW) and triterpenes content of each callus culture was measured in three weeks. The experiments were conducted in 10 replicates and repeated thrice.

The cell suspension cultures established was maintained in the MS medium (pH 5.7) supplemented with B5 vitamins, 2.0 mg L^{-1} of 2,4-D and 1.0 mg L^{-1} of kinetin. In order to obtain a suitable and homogenous growing cell, subculturing and sieving filtration of cells were repeated every ten days. Subculturing involves the transfer of 5 mL batch suspension cultures into 20 mL of fresh liquid medium. Stock cultures were aseptically prepared for treatment purposes by transferring 10 mL of cells from above culture (ten days in age) into 90 mL fresh medium in 250 mL Erlenmeyer flasks. After incubating for ten days, the stock culture was used as inocula. For each treatment, a 5 mL of cells from stock cultures were added into 20 mL of fresh liquid medium. The treatments were repeated thrice with five replicates each. The twelve days old cells were harvested for the determination of triterpenes content. To obtain the dry weight, the suspension cells were filtered through Buchner funnel using a layer of Whatman No. 1 filter paper and the cells were oven-dried at 50°C until the weight is constant.

Determination of triterpenes content: The four standard triterpenes, asiatic acid, madecassic acid, asiaticoside and madecassoside were obtained from Extrasynthese®, France. For the preparation of stock solutions of these standards, a 1.0 mg of each triterpenes was dissolved in 1 mL of absolute HPLC grade methanol (Fisher Chemicals Co., USA). The standards prepared were kept in a screw cap small dark bottle (2 mL) and refrigerated at 4°C. The asiaticoside, madecassoside, asiatic acid and madecassic acid from the stock solutions were also mixed carefully at the ratio of 2:2:1:1 (v/v/v/v). A 20 μL of individual and the mixed standards were injected into the injector using a Hamilton syringe. The retention time obtained for each standard was compared with that determined in the mixed standards (Fig. 3).

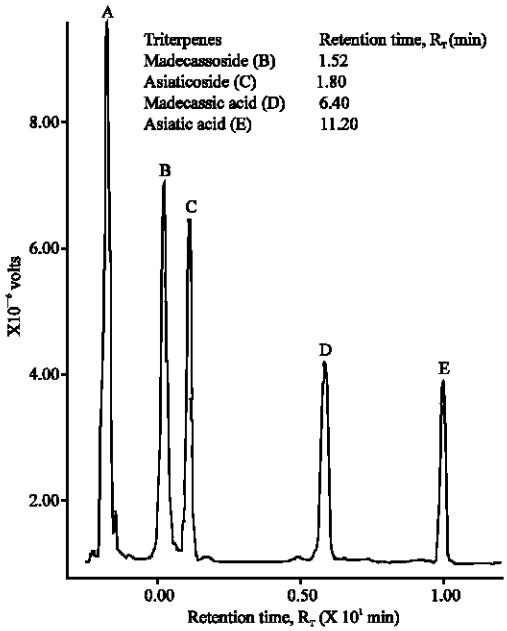

Fig. 3: A HPLC Chromatogram of mixed standard of triterpenes spectra detected by Waters 486 UV tunable absorbance detector at 206 nm using methanol: acetonitrile: water (12:1:7, v/v/v) solvent system at the flow rate of 1.0 mL/minute. (A=solvent, B = Madecassoside, C = Asiaticoside, D = Madecassic acid and E= Asiatic acid)

Extraction and analysis: The triterpenes extraction methods used was a slight modification from Inamdar *et al.*[1]. The *C. asiatica* samples were oven-dried at 50°C before been ground with mortar and pestle. A total of 1 g dried, finely powdered tissue was extracted with 20 mL of absolute analytical grade methanol (Merck, Germany) thrice and kept for one week at room temperature under dark condition. The samples were then filtered and the filtrate was evaporated to dryness using rotary evaporator (Eyela, Japan) to obtain a dark brown

crude extract. The dried crude extract was dissolved in 1.0 mL of absolute HPLC grade methanol. The extracts were finally filtered through a Waters Sep-Pak Classic Cartridge (Water, USA) prior to HPLC analysis. The filtered clear extract was placed in 1.5 mL eppendorf tubes. The extraction procedure in this study was done using methanol because preliminary extraction tests by Verma *et al.*[13] showed that methanol is more efficient in asiaticoside extraction compared to ethanol.

The three weeks old harvested calli were oven-dried for 48 h at 50°C until the dry weight is constant prior to grinding using mortar and pestle. Two grams of pooled powder from each treatment were extracted and contents of triterpenes were analyzed. Each analysis was repeated thrice.

Preparation of triterpenes precursors: The four triterpenes precursors namely, squalene, IPP, FPP and leucine used were of the highest available purity. All the precursors studied were microfilter sterilized using 0.2 μm polyethersulfone membrane (Whatman) because most of the precursors are heat sensitive. Hence, autoclave process is inappropriate. The supplementation of all the sterile precursors was carried out at the time of inoculation of callus and cells.

For leucine, a stock solution of 1 mg mL^{-1} was prepared by dissolving 10 mg leucine (Sigma, USA) with 10 mL of distilled water and was filter sterilized using 0.2 μm polyethersulfone membrane (Whatman). The corresponding concentrations of leucine were then added directly to the autoclaved culture media. Meanwhile, squalene was dissolved in distilled water prior to microfilter sterilization. A few drops of ethanol were added to help in dissolving the squalene. The IPP and FPP that separately dissolved in methanol and aqueous NH_4OH at the ratio of 7:3 with the stock concentration of 1 mg mL^{-1} were acquired from Sigma, USA. Similar to leucine, both of the stock solutions were microfilter sterilized separately before added into the media. Equal amounts of distilled water, with and without ethanol were added to the control cultures in studying the effects of squalene whereas in IPP and FPP treatments, control cultures contained equal amounts of methanol and aqueous NH_4OH at the ratio of 7:3.

Statistical analysis: The results were compared by one-way Analysis of Variance (one way ANOVA) and tested by Duncan's Multiple Range Test (DMRT) to determine the differences between treatment means at 95% ($p<0.05$) significance level. Statistical analysis was performed using the SPSS for windows version 10.0 computer statistic program.

RESULTS AND DISCUSSION

Triterpenes content in intact plant and *in vitro* cultures of *C. asiatica*: Among the four different types of samples, *in vitro* shoot cultures was found managed to produce the highest amount of asiatic acid, madecassic acid, asiaticoside and madecassoside (Table 1). *In vitro* shoot culture is supplied with a rich nutrient source and it is free from any disadvantages caused by geographical location, climate, soil and fertilization and space[14]. Thus, this could possibly explain the reason of high triterpenes content in this sample.

Meanwhile, no significant difference in terms of asiatic acid content was detected in the callus and cell suspension cultures. Similarly, intact whole plant tissues and *in vitro* whole plant cultures produced almost the same amount of madecassic acid content. Asiaticoside content in intact whole plant tissue on the other hand was 60.1% lower than that detected in the *in vitro* whole plant cultures though its content (0.382±0.054 mg g^{-1} DW) was still higher than that attained in both callus and cell suspension cultures. The content of madecassoside was found to be highly lacking in the intact whole plant tissues compared to the *in vitro* whole plant cultures. The amount of madecassoside produced in intact whole plant tissue was only 0.336±0.002 mg g^{-1} DW, value even lower than the 0.773±0.013 mg g^{-1} DW that obtained in the callus cultures.

Generally, among the four samples investigated, cell suspension cultures of *C. asiatica* produced the lowest amount of triterpenes. Callus cultures are usually more productive than cell suspension cultures of the same origin, cultivated on the same medium composition[9]. Possible secretion of the triterpenes into the surrounding medium as well as the degradation of the product could be the contributing factors for low triterpenes yield in cell suspension cultures of *C. asiatica*. This has been shown in *Alpinia officinalis* culture where 29% of oleanolic acid glycosides are transported outside the leaf protoplast through cell membrane[15]. Based on the results of these analyses, it was evident that the culture cells of *C. asiatica* also managed to produce phytochemicals but at a lower level that was much lower than in intact tissues like in the production of kavapyrones by *Piper methysticum* cells[16]. The total triterpenes produced in callus and cell suspension cultures was 3.6 and 20.2 times lower than the intact whole plant, respectively. This production percentage is higher than that reported in cell suspension cultures of *Camptotheca acuminate*, which produced camptothecin and 10-hydroxycamptothecin 100 times lower than in the original plant materials[17]. The low secondary metabolite production in culture cells

Table 1: Comparison of triterpenes content in four months old intact plant, *in vitro* shoot cultures, three weeks old leaf derived callus and twelve days old cell suspension cultures of *C. asiatica*

	Asiatic acid (mg g^{-1} DW)(%)	Madecassic acid (mg g^{-1}DW)(%)	Asiaticoside (mg g^{-1} DW)(%)	Madecassoside (mg g^{-1}DW)(%)	Total triterpenes (mg g^{-1} DW)
Whole plant (intact plant)	1.523±0.097(47.8)	0.943±0.087(29.6)	0.382±0.054(12.0)	0.336±0.002(10.6)	3.184±0.240
Whole plant (*in vitro* shoot culture)	1.802±0.054(38.1)	0.988±0.032(20.9)	0.971±0.045(20.6)	0.965±0.045(20.4)	4.726±0.176
Callus culture	0.014±0.001(1.6)	0.039±0.002(4.4)	0.058±0.041(6.6)	0.773±0.013(87.4)	0.884±0.057
Cell suspension culture	0.012±0.001(7.6)	0.005±0.001(3.2)	0.084±0.001(53.2)	0.057±0.005(36.0)	0.158±0.008

Values are means of duplicates with±SE (n=3), DW = Dry Weight

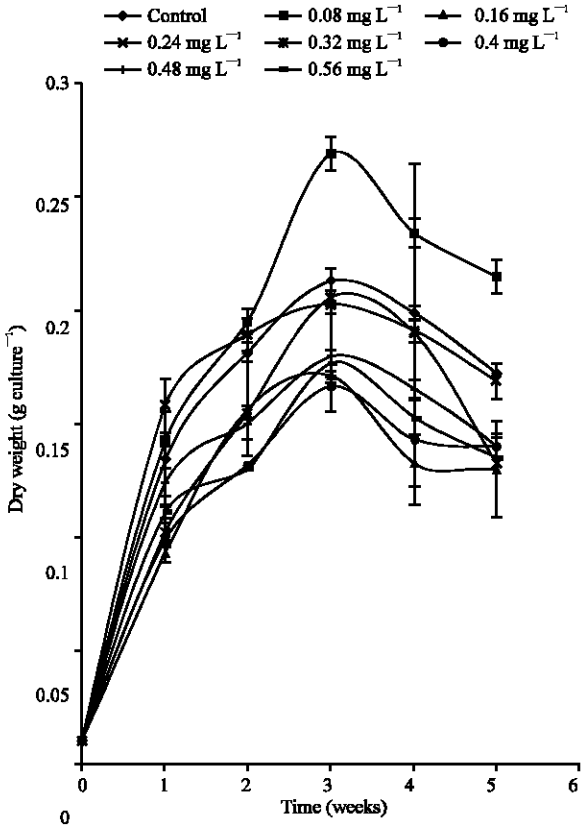

Fig. 4: Effects of squalene on the biomass production in callus cultures of *C. asiatica*. Bar indicates the SE of mean (n =3)

could be due to the lack of single specialized cell, cell compartments and tissues or specialized part of organs that serve as the synthesis and storage sites for secondary metabolites[14].

Effects of squalene: Preliminary studies on squalene at the concentrations of 0.4, 0.8, 1.2, 1.6, 2.0, 4.0, 8.0, 12.0, 16.0 and 20.0 mg L^{-1} (w/v) did not show any significant growth in terms of both fresh and dry weights compared to the control cultures after five weeks of culture. However, comparison between these concentrations tested show that 0.4 mg L^{-1} gave the highest growth (0.17±0.02 g DW $culture^{-1}$), which suggested that squalene concentrations higher than 0.4 mg L^{-1} is not suitable in obtaining higher growth for callus of *C. asiatica*. At the concentrations of 0.8 mg L^{-1} and above, toxic effects on the growth of the callus by squalene could have resulted in cells death after one week of culture. Similar toxic effect when feeding precursor at high dosage was also observed in *Catharanthus roseus* hairy root cultures and cells of *Vanilla planifolia* Andr., respectively[18,19].

The experiment was repeated using lower concentrations of squalene. Figure 4 represents the growth of the callus treated with 0.08, 0.16, 0.24, 0.32, 0.40, 0.48 and 0.56 mg L^{-1} (w/v) of squalene. From all the concentrations examined, only 0.08 mg L^{-1} managed to produce growth higher than the control. These calli achieved the highest dry weight of 0.27±0.01 g DW $culture^{-1}$ after three weeks of culture. The dry weight attained is a 1.3 fold increase compared to the control. The data obtained also revealed that there was no significant difference in terms of the biomass production between the control calli and the calli treated with 0.24, 0.32, 0.16, 0.4, 0.48 and 0.56 mg L^{-1} of squalene. This result is in agreement with the precursor feeding studies on *Comptotheca acuminate* cell lines[20].

The presence of squalene at 0.16 mg L^{-1} managed to increase madecassoside production until 1.291±0.053 mg g^{-1} DW, which was two-folds higher than that detected in the control (Table 2). The highest yields of asiaticoside as well as madecassic acid were also significantly increased in 0.16 and 0.56 mg L^{-1} squalene, respectively. The highest asiaticoside and madecassic acid contents attained in the squalene treatments were 0.162±0.018 and 0.052±0.004 mg g^{-1} DW, respectively. The effectiveness of triterpenes accumulation in callus cultures of *C. asiatica* could be due to the limiting factor of the flux whereby an exogenous supply of a biosynthetic precursor to the culture medium might improve alkaloid accumulation if the endogenous level of these precursors is a limiting factor of the flux[21]. Besides, squalene is very close to the end of the triterpenes biosynthetic pathway and it can give rise and converted to the final product more easily. There are several reports on triggering secondary metabolite production

Table 2: Effects of squalene on asiatic acid, madecassic acid, asiaticoside and madecassoside production in callus cultures of C. *asiatica*

Concentrations of squalene (mg L^{-1})	Asiatic acid (mg g^{-1} DW)	Madecassic acid (mg g^{-1}DW)	Asiaticoside (mg g^{-1} DW)	Madecassoside (mg g^{-1}DW)
Control	0.001±0.001a	0.039±0.002c	0.058±0.014b	0.773±0.018c
0.08	0.001±0.01a	0.025±0.005b	0.104±0.022c	0.624±0.143b
0.16	-	0.010±0.002a	0.162±0.018d	1.291±0.053d
0.24	-	0.011±0.004a	0.093±0.041c	0.908±0.123cd
0.32	-	0.046±0.009d	0.084±0.031bc	0.898±0.055c
0.40	-	0.017±0.001a	0.056±0.044b	0.508±0.085b
0.48	-	0.017±0.001a	0.055±0.036b	0.311±0.089a
0.56	-	0.052±0.004e	0.043±0.032a	0.277±0.124a

Values are means of duplicates with±SE (n=3), In the same column, means denoted with the different letters are significantly different at $p<0.05$ by DMRT
DW = Dry Weight, - = Not detectable

Table 3: Effects of Isopentenyl Pyrophosphate (IPP) on asiatic acid, madecassic acid, asiaticoside and madecassoside production in callus cultures of C. *asiatica*

Concentrations of IPP(mg L^{-1})	Asiatic acid (mg g^{-1} DW)	Madecassic acid (mg g^{-1}DW)	Asiaticoside (mg g^{-1} DW)	Madecassoside (mg g^{-1}DW)
Control	0.001±0.001a	0.039±0.002a	0.058±0.041c	0.773±0.013c
1.0	-	0.100±0.015d	0.011±0.121a	0.759±0.089c
2.0	-	0.134±0.005d	0.029±0.005ab	0.847±0.099d
3.0	-	0.095±0.005cd	0.077±0.042d	0.365±0.072b
4.0	-	0.068±0.009b	0.062±0.018c	0.327±0.041b
5.0	-	0.083±0.005c	0.033±0.014b	0.170±0.018a

Values are means of duplicates with±SE (n=3), In the same column, means denoted with the different letter(s) are significantly different at $p<0.05$ by DMRT
DW = Dry Weight, - = Not detectable

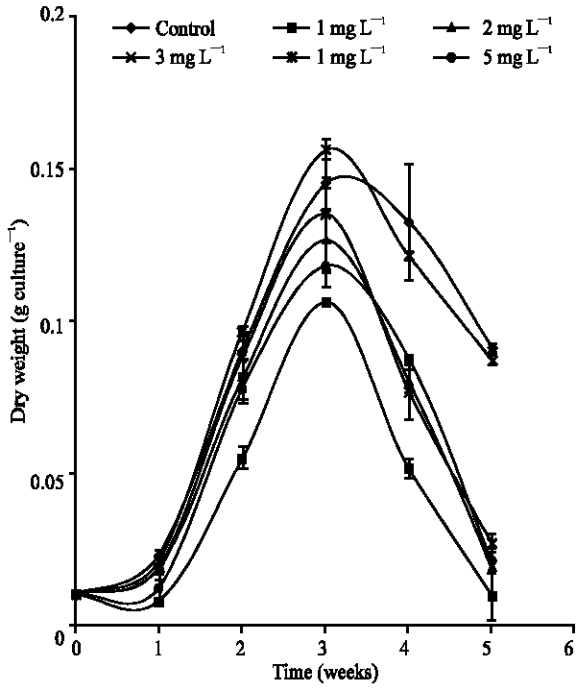

Fig. 5: Effects of Isopentenyl Pyrophosphate (IPP) on the biomass production in callus cultures of *C. asiatica*. Bar indicates the SE of mean (n =3)

by precursor feeding. For example, feeding of the iridoid loganin, the immediate precursor of secologanin, consistently resulted in enhanced terpenoid indole alkaloid accumulation by transgenic *Catharanthus roseus* cell lines[22].

Asiatic acid production, however, was not triggered by any concentrations of the squalene tested as it was only detected in minute amount in callus treated with 0.08 mg L^{-1} squalene. The entire concentrations did not show any presence of asiatic acid. Unsuccessful enhancement by precursor feeding was also evident in cell suspension culture of *Camptotheca acuminate*. Feeding with the precursors did not result in the formation of strictosidine and/ or other alkaloids[20]. The failure to accumulate asiatic acid could be due to the compartmentation of pathway enzymes in callus culture of *C. asiatica* that blocked the formation of asiatic acid. Subcellular compartmentation that allows the plant cell to separate the enzymes from their substrates and end products is also an important factor in the regulation of alkaloid metabolism[23].

Effects of Isopentenyl Pyrophosphate (IPP): Similarly, in the treatment using IPP, only IPP at the concentration of 4.0 mg L^{-1} managed to attain biomass higher than that in the control as shown in Fig. 5. The biomass obtained in 4.0 mg L^{-1} treated calli was 0.16±0.01 g DWculture^{-1}, which was also the highest biomass produced among all the IPP treatments. Meanwhile, no significant difference in terms of biomass yield was detected at 3.0, 2.0, 5.0 and 1.0 mg L^{-1}. The yields of the respective treatment were 0.14±0.02, 0.12±0.01, 0.12±0.01 and 0.11±0.01 g DW culture^{-1}. Findings in this study were similar to that obtained in *Holarrhena antidysenterica*, which growth was not affected by feeding of precursors[24].

Among all the concentrations tested, it was found that at 2.0 mg L^{-1} of IPP, the yields of both madecassoside and madecassic acid was rather high compared to the control. At 2.0 mg L^{-1}, the gain of

Table 4: Effects of Farnesyl Pyrophosphate (FPP) on asiatic acid, madecassic acid, asiaticoside and madecassoside production in callus cultures of C. *asiatica*

Concentrations of FPP (mg L^{-1})	Asiatic acid (mg g^{-1} DW)	Madecassic acid (mg g^{-1}DW)	Asiaticoside (mg g^{-1} DW)	Madecassoside (mg g^{-1}DW)
Control	0.001±0.001a	0.039±0.002c	0.058±0.014c	0.773±0.043c
1.0	-	-	0.012±0.010ab	0.659±0.015c
2.0	-	0.013±0.004a	0.006±0.019a	0.389±0.087b
3.0	-	0.035±0.004c	0.016±0.002b	0.385±0.10b
4.0	0.003±0.001a	0.052±0.011d	0.007±0.008b	0.287±0.048a
5.0	0.008±0.001b	0.027±0.005b	0.006±0.016a	0.257±0.098a

Values are means of duplicates with±SE (n=3), In the same column, means denoted with the different letter(s) are significantly different at $p<0.05$ by DMRT
DW = Dry Weight, - = Not detectable

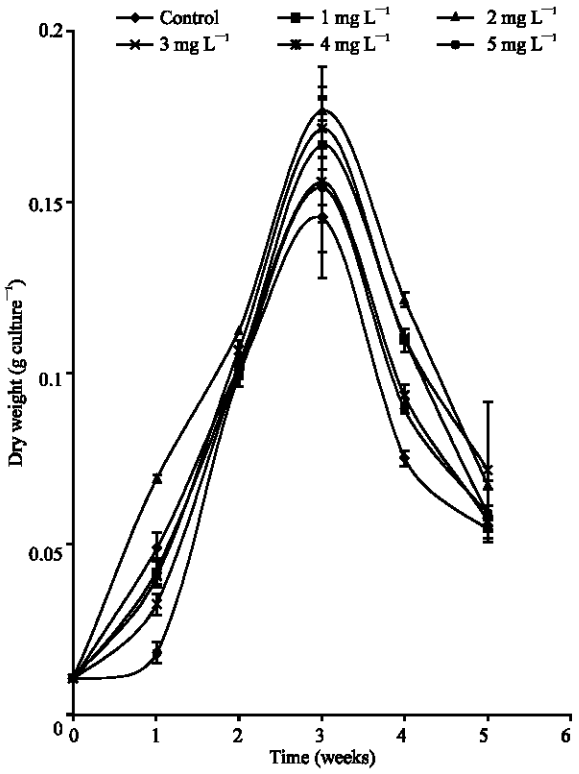

Fig. 6: Effects of farnesyl pyrophosphate (FPP) on the biomass production in callus cultures of *C. asiatica*. Bar indicates these of mean (n =3)

madecassoside was 0.847±0.099 mg g^{-1} DW, 9.6% higher than the control whilst 3.5 folds of increase to 0.134±0.005 mg g^{-1} DW of madecassic acid was produced. Meanwhile, a two fold increase in asiaticoside production was observed in 3.0 mg L^{-1} of IPP compared to the control cultures(Table 3). Stimulation of secondary metabolites by precursor feeding was also observed in *Vitis vinifera* cell suspension cultures. In this culture, the anthocyanin content was 1.2 times greater in the presences of the precursor, L-phenylalanine compared to the culture without precursor[25]. However, asiatic acid production was not triggered in any of the IPP concentrations tested. This could be due to the absence of some enzyme activities that responsible for the biosynthesis of asiatic acid in callus cultures of *C. asiatica*. Besides, limitations of the effects of the IPP on asiatic acid production may be resulted from the competition by other synthetic pathways for the same chemical as IPP is also the intermediate precursor for the biosynthesis of some secondary metabolites such as carotenoids, chlorophyll, monoterpenes and diterpenes compounds[10].

Effects of Farnesyl Pyrophosphate (FPP): As for FPP, the data obtained after five weeks of observation revealed that the highest callus growth was achieved in 2.0 mg L^{-1} (0.18±0.01 g DW culture^{-1}) at the third week of culture (Fig. 6). This was followed by the callus treated with 3.0 mg L^{-1} (1.71±0.23 g DW culture^{-1}), 1.0 mg L^{-1} (1.67±0.07 g DW culture^{-1}), 4.0 mg L^{-1} (1.56±0.27 g DW culture^{-1}) and 5.0 mg L^{-1} (1.54±0.10 g DW culture^{-1}). FPP treated callus at any of the concentrations tested managed to produce biomass higher than the control cultures. Comparable observation was also detected in cell suspension cultures of *Colchicum autummale* treated with *p*-coumaric acid and tyramine. The feeding of these precursors led to a 14 to 17 fold of increase in the cell biomass over the control culture[26].

Data obtained on effects of FPP on triterpenes production disclosed that all the concentrations studied were not suitable for enhancing asiaticoside and madecassoside yield in callus cultures (Table 4). However, the production of madecassic acid and asiatic acid was significantly increased in 4.0 and 5.0 mg L^{-1} of FPP, respectively. An enhancement of 1.4 fold was observed in madecassic acid production whilst the highest asiatic acid yield was 0.008±0.001 mg g^{-1} DW, five-times higher than that detected in the control. This result suggested that FPP might be an important precursor, which promoted madecassic and asiatic acid production. Lower amount of asiaticoside and madecassoside in callus treated with IPP probably due to a smaller breakdown rate of the asiatic acid and madecassic acid to form the glycosides. In transgenic cell line of *Catharanthus* roseus, a larger breakdown rate in line T22 increased the terpenoid indole alkaloid accumulation[22].

Table 5: Effects of leucine on asiatic acid, madecassic acid, asiaticoside and madecassoside production in callus cultures of *C. asiatica*

Concentrations of leucine (mg L^{-1})	Asiatic acid (mg g^{-1} DW)	Madecassic acid (mg g^{-1}DW)	Asiaticoside (mg g^{-1} DW)	Madecassoside (mg g^{-1}DW)
Control	0.001±0.001a	0.039±0.002d	0.058±0.041b	0.773±0.013e
1.0	0.004±0.001ab	0.020±0.005c	0.003±0.015a	0.089±0.065c
2.0	0.006±0.002b	0.002±0.001a	0.004±0.010a	0.054±0.012b
3.0	0.003±0.001a	0.006±0.001b	0.003±0.003a	0.100±0.054d
4.0	0.002±0.001a	0.003±0.002a	0.004±0.004a	0.024±0.016a
5.0	-	0.001±0.001a	0.003±0.001a	0.029±0.015a

Values are means of duplicates with±SE (n=3), In the same column, means denoted with the different letter(s) are significantly different at $p<0.05$ by DMRT
DW = Dry Weight, - = Not detectable

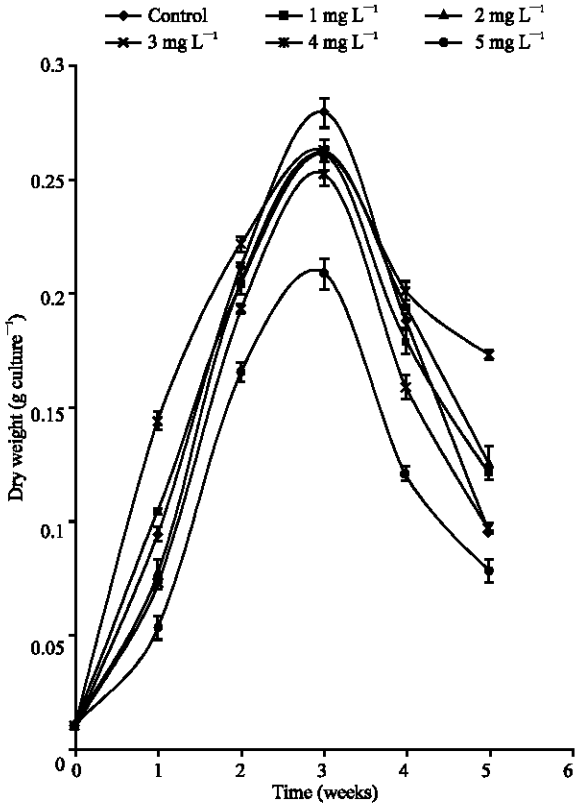

Fig. 7: Effects of leucine on the biomass production in callus cultures of *C. asiatica*. Bar indicates the SE of mean (n =3)

Effects of Leucine: Leucine is another precursor studied, as it is an important element in the biosynthesis of Acetyl-CoA that lead to the production of other intermediates in the triterpenes biosynthetic pathway. Among all the concentrations tested, it was found that the control (without leucine) managed to produce callus with high biomass growth followed by 3.0, 2.0, 1.0, 4.0 and 5.0 mg L^{-1} as illustrated in Fig. 7 The biomass growth obtained in the control culture was 0.28±0.01 g DW $culture^{-1}$. Studies had also been carried out using concentrations lower than 1.0 mg L^{-1}. However, the concentrations investigated did not promote callus growth as high as that obtained in the study using concentrations of leucine higher than 1.0 mg L^{-1}. Similar finding has been in *Catharanthus roseus* cells as non fed cultures gave the highest dry weight after one day of feeding[27]. However, the effect of feeding precursors on growth varies depending on the cell line and the type of precursor.

The effects of leucine at various concentrations on triterpenes production were also examined. From all the leucine concentrations tested (1.0 to 5.0 mg L^{-1}), it was found that none of the concentrations were suitable in enhancing triterpenes production in callus cultures (Table 5). Production of asiatic acid, madecassic acid as well as asiaticoside was inhibited following the feeding of leucine. They were present only in trace amounts. Madecassoside content in the meantime was not significantly altered by treatments using any leucine concentrations compared to the control. This study also revealed that the early intermediates in triterpenes biosynthesis are not preferred for stimulating triterpenes production in cultured cells. The same observation was reported whereby, the feeding of mevalonic acid, an early precursor of the terpenoid pathway did not affect the indole alkaloids production in *Catharanthus roseus*[28]. Another example is in the cell cultures of *Cinchona* 'Robusta' in which the addition of glyceraldehydes, an early precursor in the biosynthesis of anthraquinone did not influence the anthraquinone accumulation[7].

Leucine used in this study is the early intermediates and it did not significantly increase the triterpenes accumulation compared to the later intermediates, squalene. One possible reason for the failure of secondary metabolites stimulation could be that the early precursor is a distant precursor, which might be channeled to other pathways to form the other related compounds such as monoterpenes, indole alkaloids and sesquiterpenes[7]. Another possible reason could be that it is not a limiting factor of the triterpenes flux. Thus, triterpenes precursor feeding in this study indicates that the rate-limiting enzyme may lie between FPP and squalene.

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