SYNERGISM EFFECT BETWEEN INOCULUM SIZE AND AGGREGATE SIZE ON FLAVONOID PRODUCTION IN CENTELLA ASIATICA (L.) URBAN (PEGAGA) CELL SUSPENSION CULTURES

Suat-HianTan[1], Maziah Mahmood[2], Arbakariya Ariff [3]

[1] *Doctor, Faculty of Industrial Science and Technology, University Malaysia Pahang, Lebuhraya Tun Razak, 26300 Gambang, Kuantan, Pahang Darul Makmur,* ***tshian@ump.edu.my***

[2] *Professor Doctor, Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia,* ***maziahm@biotech.upm.edu.my***

[3] *Professor Doctor, Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia,* ***arbarif@upm.edu.my***

Abstract

The present study aimed to investigate the effect of different culture conditions on biomass content and flavonoid production of the elite C. asiatica accession UPM03. When 0.1 g inoculum 25 mL^{-1} of cell was grown in Murashige & Skoog (MS) medium supplemented with 2 mg 2,4-D l^{-1} and 1 mg kinetin l^{-1}, flavonoids were not significantly produced in cells or released into the culture medium. Production of flavonoid was correlated with the aggregation size and inoculum density. At aggregate size of 250-500 µm with 0.3 g inoculum density 25 mL^{-1}, it gave the highest luteolin content with 35.45 µg g^{-1} DW. After investigating the effect of culture conditions, i.e. pH, inoculum density, light irradiation and plant growth regulator, we found that with the optimized condition (i.e. 250-500 µm aggregate size, 0.5 g wet cell 25 mL^{-1} supplemented with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin at pH 5.7 under 16h photoperiod) the luteolin content was 11-fold higher than the cell suspension cultured at an inoculum size of 0.3 g wet cell 25 mL^{-1} with 250-500 µm aggregate size.

Index Terms: *Centella asiatica, flavonoid, light irradiation, pH regime, plant growth regulators .*

----------***----------

1. INTRODUCTION

Centella asiatica is an important medicinal herb and commercially grown in Malaysia. This herbaceous plant has widely been used for wound healing, memory improvement, treatment for mental fatigue, bronchitis, urethritis, anti-allergic and anti-cancer purposes [1]. The different uses claimed for this plant are reported to be mainly due to its high content of secondary metabolites especially flavonoids and triterpenoids [2]. The interesting biological activities of flavonoids and their low content in intact plants impose the development of alternative ways for higher production of flavonoids. Cultured plant cells are considered as known producers of secondary metabolites. Cell cultures not only have a higher rate of metabolism than differentiated plants, but also have a shorter period of biosynthetic cycles [3]. Since plant cell culture systems are relatively easy to manipulate by experimental processes, they could be developed into a large-scale culture which the secondary metabolites could be extracted. This method can supply the continuous and reliable source of natural products. Discoveries of cell culture capability in producing specific medicinal compounds at a rate similar or superior to the intact plants have accelerated in the last few years [4].

Plant cells have the natural ability to aggregate into macroscopic clumps. Xu and co-researchers [5] reported that uncontrolled clumping often causes operating problems during large-scale suspension cultures, but the tendency to aggregate can in some cases eliminate the need for artificial immobilization supports and may be exploited for large-scale phytochemical synthesis. When a smaller size of cell aggregates is preferred from the stand point of process engineering, a certain degree of cell-cell contact and cell differentiation is required for the synthesis of secondary metabolites [6].

Although for many species [7, 8] for commercial production of secondary metabolites, the low yield is one of the major problems that challenges the profitable commercialization. The morphological differentiation and biochemical processes are often linked in tissue cultures. For many plant species, the synthetic capacity of dedifferentiated cells is lower than that of the fully differentiated tissues, both quantitatively and qualitatively [9]. Chemical gradients in differentiated tissue

complex or cell aggregates encourage the synthesis of secondary metabolites. The morphological differentiation of the cell is manipulated by changing the biological, chemical and physical factors [10]. Hence, this study focused on each or different combination of the following factors, namely; the cellular/tissue differentiation status, external phytohormones, culture media and culture conditions to increase the yields of flavonoids in the cultured *C. asiatica* cell suspensions.

2. MATERIALS AND METHODS

2.1 Induction and Maintenance of Callus Cultures

In vitro C. asiatica leaf explants of the UPM03 accession were cultured on Murashige and Skoog (MS) [11] medium with B5 vitamins [12], 30 g L^{-1} sucrose, 2.75 g L^{-1} gelrite, 2 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 1mg L^{-1} kinetin to initiate callus formation and maintained at 25±2°C under 16/8h photoperiods.

2.2 Cell Suspension Cultures

The cell suspension cultures were established according to Tan et al. [13] and were maintained in the MS basal medium supplemented with 2 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin on a rotary shaker at 110 rpm. In order to obtain suitable and homogenous growing cells, sub-culturing and sieving with 750 µm pore size were carried out every 12 days. Sub-culturing was performed by transferring 5 mL of the suspension cultures into 20 mL of fresh liquid medium in 100 mL Erlenmeyer flasks. This stock culture was used as inocula.

2.3 Culture Condition Studied

The 12-day-old cell suspension was partitioned into three fractions by sieving through a stainless-steel mesh with 750 µm pore size and followed by filtration through a stainless-steel mesh with a smaller pore size of 500 µm. Finally, the filtrates were sieved through another smaller pore size of 250 µm. The first two fractions were quickly transferred back into the autoclaved media after flushing with the rinsing medium (same composition as maintenance medium) to avoid any damage of the cells.

The effects of cell inoculum size were tested, ranging from 0.1 g to 1.5 g of wet cells in 25 mL (4-60 g fresh weight per litre). For the preparation of 0.1 g wet cells 25 mL-1 inoculum size, the stock cultures were sieved through a stainless steel mesh 750 µm and 5 mL (approximately 0.10 ± 0.02 g) of the cells were then transferred into 20 mL of fresh liquid medium. To determine the accurate density, 5 mL inoculum of sieved stock cultures was centrifuged at 10,000 rpm for 15 min at 4ºC (Sigma K-20, USA). The supernatant was discarded and the fresh cell weight was determined. In the case where the fresh weight was less than 0.10 ± 0.02 g, more inoculums were added to achieve the control density. Conversely, and if the fresh cell weight was more than 0.10 ± 0.02 g, some media were added into the inoculum. A total of 5 mL inoculum containing 0.10 ± 0.02 g fresh cells was then inoculated into 20 mL of medium in a 100 mL Erlenmeyer flask. The same procedures were repeated for other inoculum sizes.

The effects of initial pH values were also tested, ranging from 3 to 9 with ph 5.7 as the control value. The pH of the media was adjusted to pH 3, 5, 5.7 and 9 with 0.01 M HCl or 0.01 M NaOH using the pH meter (Mettler Toledo FE20, Schwarzenbach, Switzerland) prior to autoclaving.

For light irradiation investigation, the cultures were incubated under either darkness or continuous exposure to light intensity of 433.33 µMol m^{-2} s^{-2}. To create a dark condition for the cultures, all the culture flasks were covered with a layer of black sugar paper.

The two PGRs chosen were 2,4-D and kinetin because they had shown potential influences both in cell growth and flavonoid production during the previous establishment of cell suspension cultures using picloram, 2,4-D, 1-Naphthaleneacetic acid (NAA), thidiazuron (TDZ), kinetin and 6-Benzylaminopurine (BAP) [13]. The concentrations of 2,4-D used were 1-3 mg/L while for kinetin was ranged from 1-2mg/L. All twelve combinations were tested in a Latin Square arrangement in order see which combination produces the optimum level of flavonoid. The control was MS medium without any hormone.

2.4 Sampling, Determination of Biomass and Residual Sugar

For sampling, three flasks were taken each time. In the cell suspension establishment, the cells were sieved with opening 750 µm after 10 days. The cells were collected on filter paper by vacuum filtration. The fresh weight of cells was determined before being dried in the oven at 50ºC until a constant weight was reached and recorded as dry weight (DW). After sampling, the residual sugar level in the supernatant was assayed by Anthrone method [14].

2.5 Measurement of Flavonoid in Cell and in Media

The media (40 mL) was concentrated by using a rotor evaporator at 40ºC. Flavonoid from the dried cells and concentrated media were extracted as described by Crozier et al. [15] and was measured according to Marinova et al. [16]. The main compound, luteolin, was quantified using HPLC method as explained by Crozier et al. [15]. For analysis of the flavonoid content, the extraction and hydrolysis conditions were based on the method described by Crozier et al. [15]. The oven-dried cells were extracted with 10 mL of 60% aqueous methanol containing 20 mM sodium diethyldithiocarbamate (NaEDTC) as an antioxidant. They were hormogenized with mortar and pestle before 2.5 mL 6 M HCl was added to each extract to give a 12.5 ml solution of 1.2 M HCl in 50%

aqueous methanol. The extracts were refluxed at 90°C for two h and known as hydrolyzed extracts. The total flavonoid content was determined by using the aluminium chloride colorimetric assay method according to Marinova et al. [16]. Luteolin was used as the standard flavonoid.

For HPLC analysis, the hydrolysed extracts were filtered through a 0.45 µm filter (Minisart RC 15, Sartorius, Germany) and analysed using the Waters (Milford, MA, USA) liquid chromatograph comprising an empowered chromatography manager, a 717 plus autoinjector, 501 HPLC pump and a 486-tunable absorbance detector. Reverse-phase separations were carried out at room temperature using a 150 x 3.9 mm I.D., 4µm C18 Nova-Pak column (Waters). The column was eluted using gradient of 22%-35% acetonitrile in water, adjusted to pH 2.5 with Trifluoroacetic Acid (TFA) at a flow rate of 0.6 mL min^{-1}. The flavonoid compound was detected at 365 nm. Identification of the compound was achieved by comparison with the commercial standard, luteolin. The quantity of flavonoid compounds in each sample was determined from the standard curve.

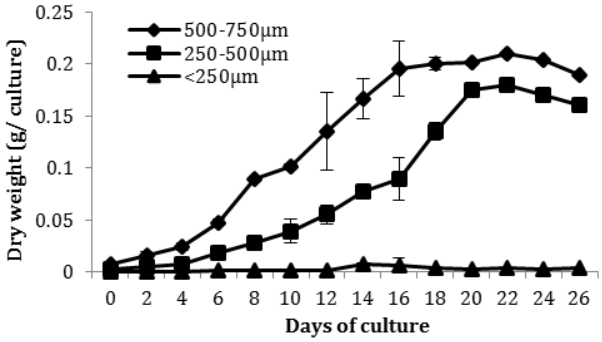
2.6 Statistical Analysis

All the experiment was run in triplicate and repeated thrice. The data were analyzed using the one-way ANOVA. The mean values were compared utilizing Duncan's multiple range test at 5% (p = 0.05) significance level, using the SPSS software version 11.5 (SPSS Inc. USA).

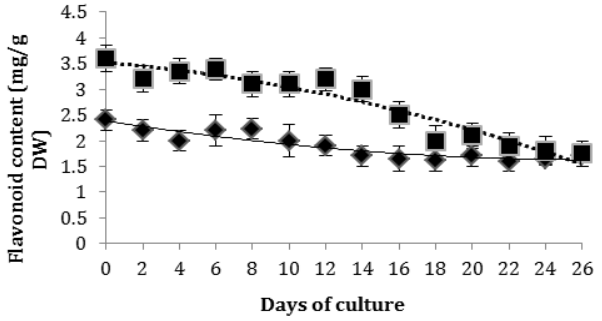
3.0 RESULTS

3.1 Effect of Aggregate Sizes and Inoculum Densities

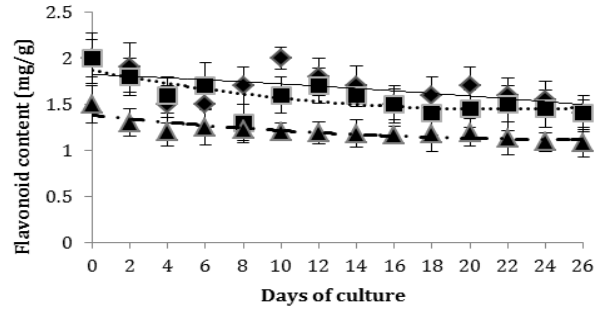
A preliminary investigation had shown that 0.1 g wet cells 25 mL^{-1} inoculum was sufficient to support the cell growth and provided a sigmoidal growth curve. However, there was no significant flavonoid production during the cultivation period until the cells went through the necrosis process where the cultures turned brown in colour (data not shown). Since it had been reported that a relative bigger size of aggregation helped to increase the secondary compound formation remarkably [5, 17, 18], thus, an investigation was carried out by using different aggregation sizes. The effects of aggregate sizes on the cell growth in the cell suspension of Malaysian *C. asiatica* accession of UPM03 are shown in Fig. 1a. For the aggregation sizes within the range of 250-750 µm, a lag phase of four days was observed. Apparently, there was no growth of the cells with the aggregate being less 250 µm. This suggested that 0.1 g wet cells 25 mL^{-1} could not support the cell growth of all the fractions during the cultivation of *C. asiatica* cell suspensions. For the aggregation sizes of 250-500 µm and 500-750 µm, the maximum cell concentrations were 0.18 g DW and 0.21 g DW, respectively. The final cell concentration was very low with a small aggregate fraction. In addition, the cell growth was greatly enhanced with the increment of aggregate fractions under 0.1 g wet cells 25 mL^{-1}. It appeared that the maximal biomass of 0.21g DW/culture obtained at the biggest aggregate fraction of 500-750 µm was about 35-fold higher compared to the minimal biomass of 0.006 g DW/culture obtained at the smallest aggregate fraction (< 250 µm).



a



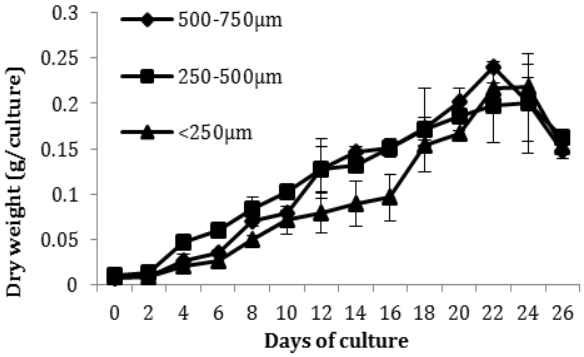
b



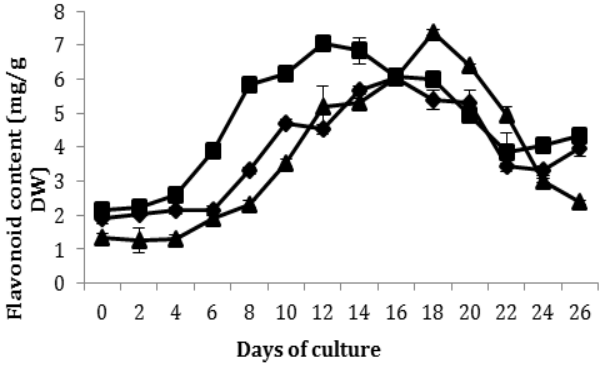
c

Fig -1: The effects of different cell aggregation sizes on the biomass accumulation (a) flavonoid production in the cell (b) and in the medium (c) of *C. asiatica* accession of UPM03. An inoculums size of 0.1 g wet cells were cultured on a 25 mL MS medium for 26 days. The bar indicates the standard deviation of mean (n=3).

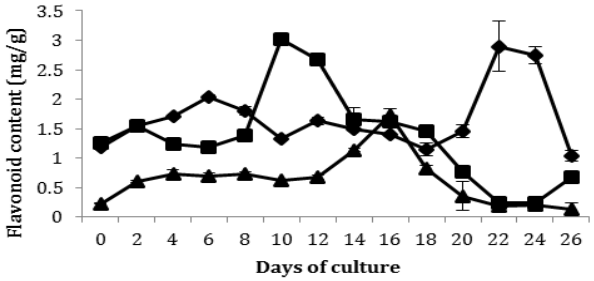
Fig. 1b and 1c show the time profiles of the flavonoid production under various aggregate fractions in the cells and the media, respectively. An increase of aggregate fractionsfrom <250 µm to 750 µm enhanced the flavonoid contents in the media. However, an opposite trend was observed in the flavonoid production on the dry weight basis. It appeared that, there were no significant differences between the flavonoid production and the cultivation period of 26 days for the various aggregate fractions either in the cells or in the media. The three aggregate fractions were found not only unable to sustain the growth of the cells but also unable to promote the flavonoid production either in the cells or the media. Thus, a further increment of inoculum size to 0.3 g wet cells 25 L^{-1} was used with the three aggregate fractions to investigate the cell growth and flavonoid production.



a



b



c

Fig -2: The effect of different cell aggregation sizes on the biomass accumulation (a), flavonoid production in the medium (c) of *C. asiatica* accession of UPM03. The inoculum size of 0.3 g wet cell were cultured in a 25 ml MS medium for 26 days.

Fig. 2a shows that 500-750 µm fractions reached the stationary growth phase on the 22nd day in the suspension culture under the described conditions, whereas 250-500 µm and <250 µm fractions have a longer growth phase up to the 24th day. Similarly, this observation was the same with the *C. asiatica* cultures using 0.1 g wet cells 25 mL^{-1} MS medium, whereby 500-750 µm was the highest biomass accumulation on the 16th day and 250-500 µm on the 20th day. During the middle of exponential stage in the suspension culture, the flavonoid content in the cells increased rapidly to high levels of 7.38 ± 0.09, 7.05 ± 0.07 and 6.02 ± 0.05 mg g^{-1} DW for the samples collected on day 18 (<250 µm), day 12 (250-500 µm) and day 16 (500-750 µm), respectively. The content reduced when the cells entered the end of the exponential phase and reached the low levels of 2.38 ± 0.07 mg g^{-1} DW (<250 µm), 3.85 ± 0.55 mg g^{-1} DW (250-500 µm) and 3.99 ± 0.26 mg g^{-1} DW (500-750 µm) on around the 22nd day (Fig. 2b). The main compound, luteolin was quantified for the samples, which have the highest flavonoid content. Luteolin differed with the aggregate size. The highest luteolin level was detected in the 250-500 µm aggregate size with 35.45 ± 2.2 µg g^{-1} DW (Table 1).

For flavonoid accumulation in the media as illustrated in Fig. 2c, the results showed that 250-500 µm of fractions attained the highest flavonoid content, i.e. 3.02 ± 0.05 mg g^{-1}, on day 10, followed by 500-750 µm (2.89 ± 0.43 mg g^{-1}) on day 22 and <250 µm (1.74 ± 0.11 mg g^{-1}) on day 16. The flavonoid accumulation patterns for the three aggregate sizes were different among themselves (Fig. 2c).

Fig. 3 shows the dynamic profiles of sucrose consumption under various aggregation sizes. The sucrose consumption corresponded to the growth of *C. asiatica*. While medium sucrose was almost consumed on day 18, the cell density reached the peak on day 22 (Fig. 2a). Both the cell growth and

sucrose consumption rates were relatively lower at an aggregate size of <250 µm, while they were found almost constant at bigger aggregate sizes. The average glucose consumption rates were 0.58, 0.59 and 0.69 g glucose l^{-1} per day, and the corresponding cell yields were 0.025, 0.018, 0.019 g cell/g glucose for <250 µm, 250-500 µm and 500-750 µm of aggregate size, respectively. No significant effect was observed on the cell yields against sucrose during the cultivation of *C. asiatica* for the bigger aggregate sizes.

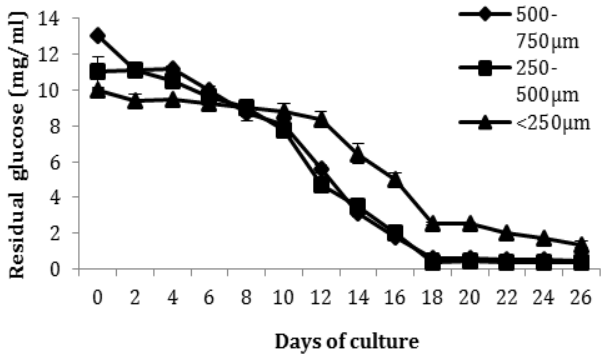
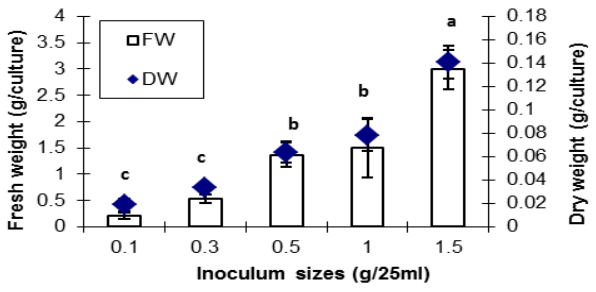


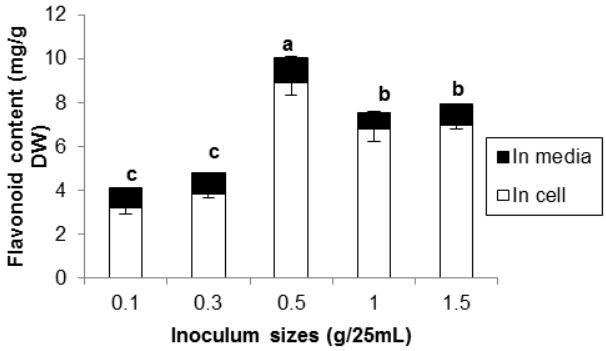
Fig -3: Sucrose consumption profiles of different aggregation sizes of *C. asiatica* accession of UPM03 cells in the suspension cultures. The inoculums size of 0.3 g wet cells were cultured in a 25 ml MS medium for 26 days.

3.2 Effect of Inoculum Densities

The effects of inoculum size within the range of 0.1 to 1.5 g wet cells 25 mL^{-1} on the *C. asiatica* cell cultures were investigated. The differences in biomass accumulation by the use of different inoculum sizes are shown in Fig. 4a for fresh and dry weight. The results revealed that the biomass cells were very low at low inoculum size and the cell growth was greatly enhanced with the increment of inoculum size from 0.1 to 1.5 g wet cells 25 mL^{-1}. The maximum biomass yields of 3.05 g FW and 0.14 g DW were obtained at inoculum size of 1.5 g wet cells 25 mL^{-1}. It appeared that the flavonoid content of cell suspensions on the dry weight basis differed remarkably with the inoculums sizes.



a



b

Fig -4: The effect of cell inoculum size on the biomass accumulation (a) and flavonoid production in the cell and media (b) of *C. asiatica* accession of UPM03. The different letters indicate that the values are significantly different ($p \leq 0.05$).

Fig. 4b. In the inoculums with the sizes less than 1 g wet cells 25 mL^{-1}, the flavonoid content increased with the inoculum sizes. However, when the inoculum sizes exceeded 1.0 g wet cells 25 mL^{-1}, the value remained the same. Likewise, a similar pattern was observed in the flavonoid production in the media. These findings indicated that large sizes of inoculum resulted in low flavonoid contents.

3.3 Effect of Initial pH Values

The cell suspension was exposed to four pH regimes. The profiles for biomass production in fresh and dry weights are shown in Fig. 5a. Both pH 5 and pH 5.7 obtained a very similar biomass concentration of 0.158 g/culture and 0.157 g/culture on the dry weight basis, respectively. Meanwhile, Fig. 5b shows the flavonoid production in different pH values intracellularlly and extracellularly, respectively. In the cells, the accumulation of flavonoids was maximum at pH 5.7, i.e. 3.658 mg g^{-1} DW. However, the extracellular flavonoid content in the media was increased gradually from pH 3 to pH 9. In the high pH value of 9, the media changed colour from clear to reddish on day 6.

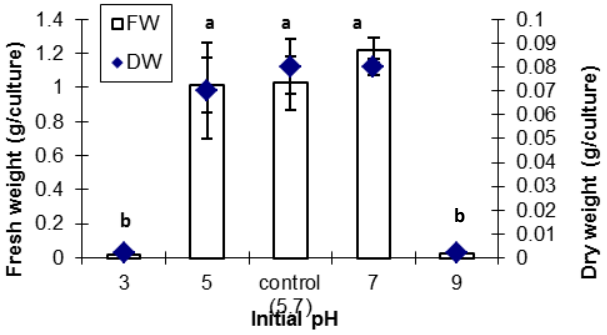
3.4 Effect of Light Irradiation

Fig. 6a illustrates that continuous high level light irradiation was found to be able to increase the cell growth significantly in both fresh and dry cell weights. Additionally, flavonoid biosynthesis was stimulated considerably by high level light irradiation in *C. asiatica* cell suspension either in the cells or in the media (Fig. 6b). The flavonoid content was stimulated almost 2.5-fold for the light-treated culture with 3.66 mg g^{-1} as compared to the dark-treated cell cultures with only 1.41 mg g^{-1}. Although the extracellular flavonoid content was comparably lower than the intracellular flavonoid content, the

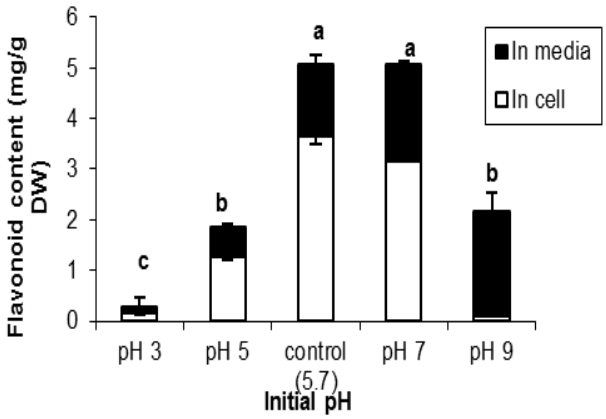
extracellular flavonoid content in the light-treated cultures was 0.79 mg g^{-1}, which showed 3.6-fold increase compared to the dark-treated cultures (0.23 mg g^{-1}).

3.5 Effect of Plant Growth Regulators

In the preliminary study on the establishment of cell suspension cultures using different auxins and cytokinins, 2,4-D and kinetin were found to be the most potential PGRs in promoting biosynthesis of flavonoids [7]. Thus, in this study, both plant growth regulators were tested at different concentrations and various combinations. Fig. 7a shows the biomass accumulation of fresh and dry weight of the cultured cells obtained under different combinations of 2,4-D and kinetin. When the concentration of 2,4-D was increased in the treatment, the cell growth was decreased. On the contrary, with the increased concentration of the kinetin, the cell growth was also increased. The increase in cell growth could be due to the expansion of cells which increased the cell size to form bigger clumps. The results showed that the flavonoid production in the cells was significantly increased with the increasing concentrations of 2,4-D (Fig. 7b). Flavonoid content was significantly declined with the increasing concentration of Kinetin. Evidently, the flavonoid content increased up to 11.87 mg g^{-1} DW in the cells as well as 6.62 mg g^{-1} in the media (Fig. 7b), four-fold and 1.3-fold increments compared to the control medium when the medium was supplemented with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin, respectively. Furthermore, minimal growths of 0.07 mg g^{-1} FW and 0.063 mg g^{-1} DW were obtained. The trend demonstrated that when the cell growth decreased, the flavonoid content in the cells increased. However, there were no significant differences among the flavonoid content in media within all the concentrations except for the supplementation of 2,4-D and kinetin with the ratio 3:1 and 3:2. A high concentration of 2,4-D was more effective in combination with a low concentration of kinetin for enhancing the flavonoid content in *in vitro* cultures. Luteolin content was greatly enhanced to 390±30 µg/g DW in the cell when treated with 3 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin (Table -1).



a



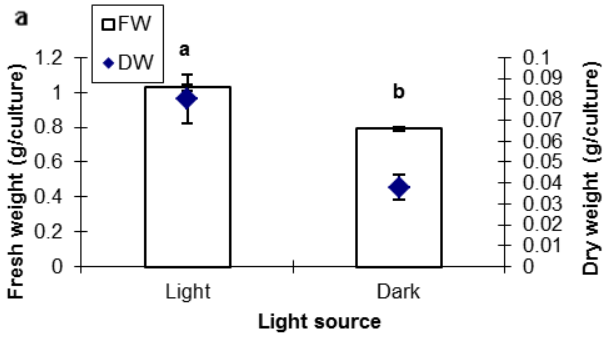
b

Fig -5: The effect of initial pH values on the biomass accumulation (a) and flavonoid production in the cell and media (b) of *C. asiatica* accession of UPM03. They were cultured in a 100 ml conical flask at an inoculums size of 0.5 g wet cells in 25ml for 12 days. The size of aggregate used was 250-500 µm. The different letters indicate that the values are significantly different ($p \leq 0.05$).

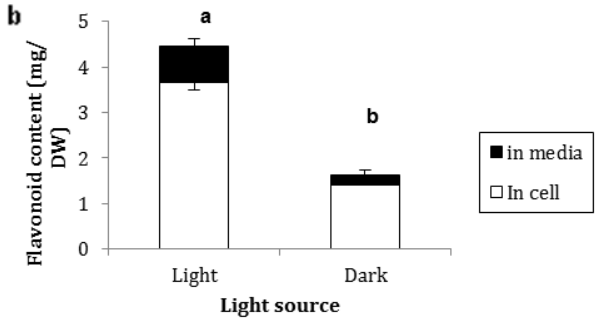
Table -1: Luteolin production in each size aggregation with it harvested day in the *C. asiatica* cell suspension cultures at pH 5.7 with a photoperiod of 16h under the fluorescent light.

Cell culture condition	Inoculum size	Luteolin production (µg/g DW)
2mg L^{-1} 2,4-D + 1mg L^{-1} kinetin + 0.3 g wet cell 25 mL^{-1}	500-750 µm (day18)	30.32 ± 2.1
	250-500 µm (day 12)	35.45 ± 2.2
	<250 µm (day 16)	20.55 ± 1.9
2 mg L^{-1} 2,4-D + 1mg L^{-1} kinetin + 0.5 g wet cell 25 mL^{-1}	250-500 µm (day 12)	110 ± 13
3mg L^{-1} 2,4-D + 1mg L^{-1} kinetin + 0.5 g wet cell 25 mL^{-1}	250-500 µm (day 12)	390±33

Each value represents the mean ± SE, n=3

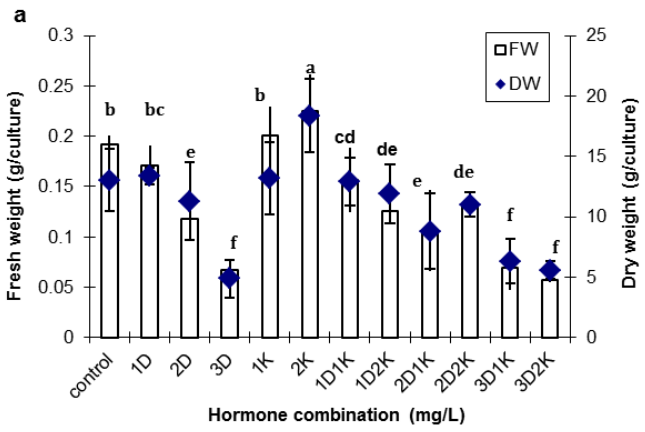


a

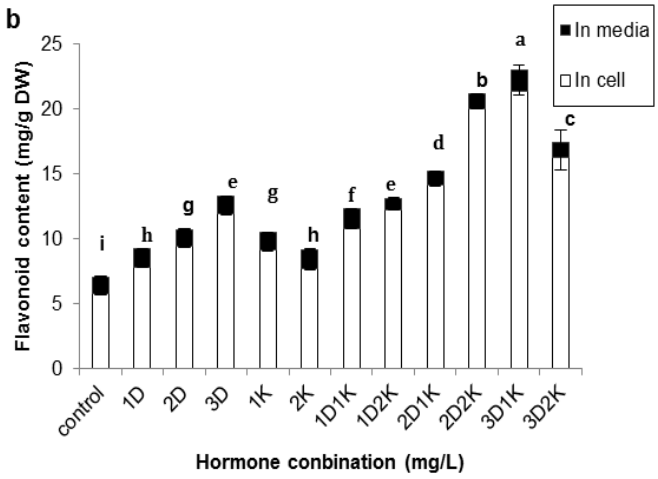


b

Fig -6: The effect of different light sources on the biomass accumulation (a) and flavonoid production in the cell and media (b) of *C. asiatica* accession of UPM03. They were cultured in a 100 ml conical flask at an inoculum size of 0.5 g wet cells in 25 ml after cultured for 12 days. The size of aggregate used was 250-500 µm. The different letters indicate that the values are significantly different ($p \leq 0.05$).



a



b

Fig -7: The effect of 2,4-D (D) and kinetin (K) in combination on the biomass accumulation (a) flavonoid production in the cell and media (b) of *C. asiatica* accession of UPM03. They were cultured at an inoculum size of 0.5 g wet cells in 25 ml after cultured for 12 days. The size of aggregate used was 250-500 µm. The different letters indicate that the values are significantly different ($p \leq 0.05$).

4. DISCUSSION

Primary metabolism is associated with the exponential phase of a culture where the sole products of metabolism are either essential for growth or the by-products of energy-yielding metabolism [19]. In most batches of cell suspension cultures, secondary metabolite accumulation tends to increase at the end of the rapid cell division in the growth cycle [20]. However, in some cases, the production of secondary products did not show a positive correlation with the maximal growth rate of the culture [19].

The early production of flavonoid during the cell cultivation was consistent with the previous studies and suggested that the secondary metabolites could be accumulated maximally during the exponential phase. Wu et al. [9] reported that the maximum salidroside yield of *Rhodiola sachalenensis* suspension cultures was 19.69 ± 1.84 mg L^{-1} salidroside on day 10 (early exponential phase) after inoculation. Additionally, Jacinda et al. [19] found that the highest concentrations of the targeted triterpenoids in the cell suspension of *C. asiatica* were observed on day 16 and day 20 for both investigated accessions at the end of the exponential phase.

It appeared that high cell density favored mass tissue formation and played an important role in the flavonoid production either in the cells or in the media as well as the

time of flavonoid accumulation. These observations might reflect a competition for metabolites utilized in the primary metabolism with those pathways leading to the formation of the secondary products [19]. A similar phenomenon also occurred in the cucurbit cell cultures for cucurbitacin production [21]. Rama et al. [22] also found that the cell aggregates at 500 µm diameter favoured the biomass accumulation, while the accumulation of berberine increased with the increase in cell aggregate size.

There was a minimum size of explants or quantity of separated cells per unit culture volume for successful culture initiation [23]. Large explants generally survive more frequently and grow more rapidly than relatively small explants at the initial stage of culture [24]. Inoculation density was found to affect the activities of individual enzymes in suspended plant cell cultures for the synthesis of secondary metabolites [25]. The higher final cell mass could be achieved in the cell culture with higher inoculum sizes. A similar phenomenon was also observed in the cell growth of *Panax ginseng* cultures [26]. Wu et al. [27] also reported that the final weight of root biomass was low when less dense inoculum sources were used, but the development was greatly enhanced with inoculum sizes of 10 to 20 g/l. Previous research studies stated that the cell growth kinetics and secondary metabolite formation in plant cell cultures were greatly affected by the inoculum size. For example, Sakurai and co-researchers [28] reported that the anthocyanin productivity in strawberry suspension culture increased in proportion to the inoculum size up to 0.25 g 25 mL^{-1}, whereas the maximum cell concentration was achieved at 0.5 g 25 mL^{-1}. The stimulatory effects of inoculum size on the secondary metabolite production were also observed in ginsenoside accumulation in ginseng suspension culture [29] as well as ajmalicine and catharanthine production in immobilized *Catharanthus roseus* cell cultures [30].

Notably, *C. asiatica* cell suspension can be maintained in a good condition when the medium pH is between 5.0 and 6.0, but the concentration of hydrogen ions in the medium changes during the development period, decreases during ammonia assimilation and increases during nitrate uptake [27]. Initial medium pH range of 5.0 to 6.0 was best in promoting the growth of *Echinacea* roots and their accumulation of phenols and flavonoids. However, the growth was inhibited at the initial pH lower than 5.0 or higher than 6.0 [27]. In addition, Shu and Lung [31] also stated that a higher culture pH might facilitate the transport of methanol-soluble metabolites from mycelia of *Antrodia camphorate* into the medium. The formation of reddish culture during high pH regime might be due to the presence of high content of anthocyanin, which was the end product in the flavonoid pathway. The intermediate flavonoid might have been secreted out into the medium for anthocyanin formation which caused lower flavonoid content in the cells. This showed that flavonoid in this culture has the tendency to be secreted out into the medium instead of retain in the cell. Hence, the metabolites presented in cells were relatively lower as compared to the media.

Light is thought to have effects on enzyme induction and activation [32]. The increment of flavonoid production in response to light in this study is similar with previous researchers who also reported that the secondary metabolites were induced by light in cell or tissue cultures of several species, such as *Rudbeckia hirta* L. [33] and *Vitis vinifera* [34]. It was reported that light enhanced the production involving the protection function of the plants against UV damage.

Notably, cytokinins are known to enhance the production of secondary metabolites and play an important role in cotydifferentiation [35]. However, externally added kinetin inhibited both salidroside accumulation and growth of CCA suspension culture [21]. In addition, Pasqua and co-workors [36] also reported that high concentrations of cytokinins reduced the anthocyanin production. On the other hand, with regard to auxins, the highest anthocyanin production was obtained in the presence of low auxin concentration, which contradicted with the finding of this present study. Nevertheless, the current result of high auxin concentration that enhanced the secondary metabolites production was found to be consistent with the studies carried out on *Tinospora cordifolia* Miers [22].

CONCLUSIONS

Cultivation of cell suspension is an efficient method for producing useful phytomolecules. We found that there is a positive relationship between the flavonoid production and the cell aggregate size. A bigger size of cell aggregation with a high inoculum density is attributed as a useful finding flavonoid production either intercellularly or extracellularly. In this study, in vitro conditions strongly affected the cell growth and accumulation of flavonoid content in C. asiatica tissues. The best performance was obtained in the MS medium supplemented with 3.0 mg L-1 2,4-D and 1 mg L-1 kinetin , 3% (w/v) sucrose, pH and the inoculum size of 0.5g wet cells 25 mL-1 at 250-500 µm aggregate size in the presence of light or without light. This particular culture protocol will prove beneficial for large-scale biomass production and secondary metabolites of C. asiatica.

ACKNOWLEDGEMENTS

One of the author, SH Tan, would like to thank Universiti Malaysia Pahang RDU120332 for financial support.

REFERENCES

[1] Zainol MK, Abd-Hamid A, Yusof S and Muse R 2003 Antioxidative activity and total phenolic compounds of

leaf,root and petiole of four accessions of Centella asiatica (L.) Urban; Food Chemistry 81, 575-581.

[2] Zheng CJ and Qin LP 2007 Chemical components of Centella asiatica and their bioactivities; Chinese Journal of Integrative Medicine 5, 348-351.

[3] Chakraborty A and Chattopadhyay S 2008 Stimulation of menthol production in Mentha piperita cell culture; In Vitro Cellullar and Developmental Biology –Plant 44, 518-524.

[4] Siahsar B, Rahimi M, Tavassoli A and Raissi AS 2011 Application of biotechnology in production of medicinal plants; American-Eurasian Journal of Agriculture and Environmental Science 11, 439-444.

[5] Xu JF, Su ZG and P.S F 1998 Suspension culture of compact callus aggregate of Rhodiola sachalinensis for improved salidroside production; Enzyme Microbiology Technology 23, 20-27.

[6] Madhusudhan R and Ravishankar GA 1996 Gradient of anthocyanin in cell aggregates of Daucus carota in suspension cultures; Biotechnology Letter 18, 1253-1256.

[7] Mathur M and Ramawat KG 2007 Guggulsterone production in cell suspension cultures of the guggul tree, Commiphora wightii, grown in shake-flasks and bioreactors; Biotechnology Letter 29, 979-982.

[8] Park JM, Yoon SY, Giles IKL, Songstad DD, Eppstein D, Novakovski D, Friesen L and Roewer I 1992 Production of sanguinarine by suspension culture of Papaver somniferum in bioreactors; Journal of Fermentation Bioengineering 74, 292-296.

[9] Wu SX, Zu YG and Wu M 2003 High yield production of salidroside in the suspension culture of Rhodiola sachalinensis; Journal of Biotechnology 106, 33-43.

[10] Edahiro JI and Seki M 2006 Phenylpropanoid metabolite supports cell aggregate formation in strawberry cell suspension culture; Journal of Bioscience and Bioengineering 102, 8-13.

[11] Murashige T and Skoog F 1962 A revised medium for rapid growth and bioassays for tobacco tissue culture; Physiology Plantarum 15, 473-497.

[12] Gamborg OL, Miller RA and Ojima K 1968 Nutrient requirements of suspension cultures of soybean root cells; Experimental Cellullar Research 50, 151-158.

[13] Tan SH, Musa R, Ariff A and Maziah M 2010 Effect of plant growth regulators on callus, cell suspension and cell line selection for flavonoid production from pegaga (Centella asiatica L. urban); America Journal of Biochemistry and Biotechnology 6, 284-299.

[14] Jermyn MA 1975 Increasing the sensitivity of the anthrone method for carbohydrate; Analytical Biochemistry 68, 332-335.

[15] Crozier A, Jensen E, Lean MEJ and McDonald M 1997 Quantitative analysis of flavonoids by reversed-phase high-perfomance liquid chromatography; Journal of Chromatography A 761, 315-321.

[16] Marinova D, Ribarova F and Atanassova M 2005 Total phenolics and total flavonoids in Bulgarian fruits and vegetables; Journal of the University Chemistry and Technology Metallurgy 40, 255-260.

[17] Fu CX, Zhao DX, Huang Y and Ma FS 2005 Cellular aggregate size as the critical factor for flavonoid production by suspension cultures of Saussurea medusa; Biotechnology Letter 27, 91-95.

[18] Zhao D, Huang Y, Jin Z, Qu W and Lu D 2003 Effect of aggregate size in cell cultures of Saussurea medusa on cell gowth and jaeosidin production; Plant Cell Reports 21, 1129-1133.

[19] Jacinda JT, Riaan M and Ian AD 2008 Characterisation of two phenotypes of Centella asiatica in Southern Africa through the composition of four triterpenoids in callus, cell suspensions and leaves; Plant Cell, Tissue and Organ 94, 91-99.

[20] Collin HA and Edwards S 1998 Secondary product synthesis by plant tissue cultures; in Plant Cell Cultures (Oxford, BIOS Scientific Publisher Ltd) pp 105-106.

[21] Halaweish FT and Tallamy DW 1998 Production of cucurbitacins by cucurbit cell cultures; Plant Science 131, 209-218.

[22] Rama RB, Vijay KD, Naga AR, Jalaja N, Vaidyanath K, Maruthi RA, Srinath R, Rathnagir IP 2008 Effect of growth regulators, carbon source and cell aggregate size on berberine production from cell cultures of Tinospora cordifolia Miers; Current Trends Biotechnology Pharmacy 2, 269-276.

[23] Thanh NT, Murthy HN, Yu KW, Jeong CS, Hahn EJ and Paek KY 2004 Effect of inoculum size on biomass accumulation and ginsenoside production by large-scale cell suspension cultures of Panax ginseng; Journal of Plant Biotechnology 6, 265-268.

[24] Paek KY, Hahn EJ and Son SH 2001 Application of bioreactors for large-scale micropropagation systems of plants; In Vitro Cellullar and Developmental Biology - Plant 37, 284-292.

[25] Gulik WMV, Nuutila AM, Vinke KL, Hoopen HJGT and Heijnen JJ 1994 Effect of carbon dioxide, air flow rate, and inoculation density on the batch growth of Catharanthus roseus cell suspensions in stirred fermentors; Biotechnology Progress 10, 335-339.

[26] Akalezi CO, Liu S, Li QS, Yu ST and Zhong JJ 1999 Combined effects of initial sucrose and inoculums size on cell growth and ginseng saponin production by suspension cultures of Panax ginseng; Process Biochemistry 34, 639-642.

[27] Wu CH, Dewir YH, Hahn EJ and Paek KY 2006 Optimization of culturing conditions for the production of biomass and phenolics from adventitious roots of Echinacea angustifolia; Journal of Plant Biology 49, 193-199.

[28] Sakurai M, Mori T, Seki M and Furusaki S 1996 Changes of anthocyanin composition by conditioned

medium and cell inoculum size using strawberry suspension culture; Biotechnology Letter 18, 1149-1154.

[29] Jeong CS, Murthy HN, Hahn EJ, Lee HL and Paek KY 2009 Inoculum size and auxin concentration influence the growth of adventitious roots and accumulation of ginsenosides in suspension cultures of ginseng (Panax ginseng C.A. Meyer) Acta Physiology Plantarum 31, 219-222.

[30] Lee C and Shuler M 2000 The effect of inoculum density and conditioned medium on the production of ajmalicine and catharanthine from immobilized Catharanthus roseus cells; Biotechnology and Bioengineering 67, 61-71.

[31] Shu CH and Lung MY 2008 Effect of culture pH on the antioxidant properties of Antrodia camphorata in submerged culture; Journal of the Chinese Institute of Chemical Engineers 39, 1-8.

[32] Jaleel CA, Gopi R, Gomathinayagam M and Panneerselvam R 2009 Traditional and non-traditional plant growth regulator alter phytochemical constituents in Catharanthus roseus; Process Biochemistry 44, 205-209.

[33] Luczkiewicz M, Zarate R, Dembinska-Migas W, Migas P and Verpoorte R 2002 Production of pulchelin in hairy roots, callus and suspension cultures of Rubeckia hirta L; Plant Science 163, 91-100.

[34] Zhang W, Curtin C, Kikuchi M and Franco C 2002 Integration of jasmonic acid and light irradiation for enhancement of anthocyanin biosynthesis in Vivis vinifera suspension cultures; Plant Science 162, 459-468.

[35] Suri SS and Ramawat KG 1995 In vitro hormonal regulation of laticifers differentiation in Calotropis procera; Annals of Botany 75, 477-480.

[36] Pasqua G, Monacelli B, Mulinacci N, Rinaldi S, Giaccherini C, Innocenti M and Vinceri FF 2005 The effect of growth regulators and sucrose on anthocyanin production in Camptotheca acuminata cell cultures; Plant Physiology Biochemistry 43, 293-298.