Chapter III

Initiation of Cell Suspension Cultures of *Achyranthes aspera* and Enhancing the Production of 20-hydroxyecdysone by Elicitor and Precursor Feeding

ABSTRACT

*Achyranthes aspera* cell suspension culture was raised in the shake flask to investigate the cell growth and production of 20-hydroxyecdysone. Cell suspension cultures were established from friable type-1 callus, initiated from leaf explants and they were cultured in liquid MS medium supplemented with combinations of 2, 4-D (1 mg L\(^{-1}\)) and NAA (1 mg L\(^{-1}\)). Cells in suspension culture grew rapidly and after 20 days of culture, the biomass accumulated was about 3.34±0.05 g FW/flask and the 20-hydroxyecdysone concentration was about 0.24 mg g\(^{-1}\) DW. Effect of methyl jasmonate (elicitor), cholesterol and 7-dehydrocholesterol (precursors) on 20-hydroxyecdysone production was also studied. When 15-day-old cultures were treated with 0.6 mM methyl jasmonate for six days, it produced 20-hydroxyecdysone 0.35 mg g\(^{-1}\) DW which was greater than that of the untreated cells. Likewise, addition of cholesterol and 7-dehydrocholesterol triggered the production of 20-hydroxyecdysone to 0.31 mg g\(^{-1}\) DW and 0.28 mg g\(^{-1}\) DW respectively.

Key words: *Achyranthes aspera*, 20-hydroxyecdysone, cell suspension culture, methyl jasmonate, cholesterol, 7-dehydrocholesterol.
3.1 INTRODUCTION

Plant cells are biosynthetically totipotent and every single cell in culture retains complete genetic information and is theoretically capable of producing a range of chemicals that are synthesized by the parent plant. Cell suspension culture systems are designed for large-scale culturing of plant cells for the controlled production of valuable secondary metabolites and other biologically active compounds. A suspension culture is normally derived by transferring friable portions of the callus clumps into a liquid medium. It is then sustained in appropriate conditions of aeration, agitation, temperature, light and other physical parameters (Chattopadhyay et al. 2002). The suspension culture contains single cells, small cell groups as well as larger cell aggregates dispersed in a liquid medium displaying considerably higher rates of cell division than cells in callus culture. The greatest advantage of this method is that it ultimately provides a reliable source of natural products (Rao and Ravishankar, 2002). It offers easiness in product isolation and purification, mainly in the case when the product is released into the culture medium (Cai et al. 2012). Meanwhile, plant cell and tissue culture technology has gradually replaced the whole plant cultivation as a source of secondary metabolites. There are many fruitful examples of tissue culture of medicinal plants where an improvement in the productivity of the bioactive secondary metabolites has been achieved.

In many cases, established cell cultures from many plants do not synthesize adequate amounts of the required secondary metabolites, moreover; current yield and productivity cannot fulfil the commercial demand. In recent years, several strategies have been adopted to assess biomass accumulation and to stimulate the formation of secondary metabolites. These include the improvement of strain, optimization of
culture environments and medium, elicitation, nutrient and precursor feeding, immobilization, and biotransformation methods.

Plants and in vitro cultured plant cells, display morphological and physical reactions to microbial, physical or chemical factors called as ‘elicitors’. An ‘elicitor’ might be described as a substance, when it is introduced in minor concentrations to a living cell system, is capable of initiating or enhancing the biosynthesis of specific metabolites. Elicitation is the induced biosynthesis of specific compounds due to the application of trace amounts of elicitors (Radman et al. 2003). On the basis of their nature elicitors are divided into abiotic elicitors or biotic elicitors. Biotic elicitors are substances of biological origin, frequently originated as an effect of fungi, bacteria, virus or herbivore infections (exogenous elicitors), in certain cases they are released by the attacked plant due to the action of enzymes of the pathogen (endogenous elicitors) (Angelova et al. 2006). Habitually, complex biological preparations are used as elicitors and the molecular structure of the active ingredients is unidentified (Angelova et al. 2006). Abiotic elicitor or stress agents are of non-biological origin, which includes various types of inorganic salts and also physical factors like UV radiation, heavy metal salts (Cu and Cd ions), Ca$^{2+}$, high pH etc. (Eilert, 1987; Radman et al. 2003).

Precursor feeding is another approach adopted to increase the yield of the desired product in plant cell cultures. It is based on the knowledge that any compound, which is an intermediate, in or at the beginning of a secondary metabolite biosynthetic route, has a good chance of enhancing the yield of the final product. Efforts to induce or increase the synthesis of plant secondary metabolites, through providing precursor or intermediate compounds, have been effective in many cases (Moreno et al. 1993; Whitmer et al. 1998).
Biotechnological techniques, comprising of plant cell cultures appears to be a good alternative for synthesis of ecdysteroids of scientific and practical interest. *In vitro* cultures are regarded as feasible routes for the production of biologically valuable phytoecdysteroids and cell cultures are considered as the amenable experimental model for studies regarding biosynthesis, regulation and physiological role of phytoecdysteroids. The capability of several plant species to produce phytoecdysteroid *in vitro* was first described for the callus tissues of *Achyranthes faurieri* (Hikino et al. 1971) and *Trianthema portulacastrum* (Ravishankar and Mehta, 1979). Analysis of ecdysteroid profiles in *Ajuga turkestanica* callus and suspension cultures established, their ability to synthesize 20-hydroxyecdysone two to six fold higher level than the level found in aerial parts and roots of this plant (Lev et al. 1990). Moreover, cell cultures of *Serratula tinctoria* (Corio-Costet et al. 1996) remain as an important source of ecdysteroids. Likewise, the probability of obtaining substantial amounts of 20-hydroxyecdysone, which has potential applications in human medicine (Lafont and Dinan, 2003), along with other rarer ecdysteroid analogues, containing interesting biological properties is found to be promising (Báthori and Pongrácz, 2005) from other plant species.
3.2 **SPECIFIC OBJECTIVES**

The objectives of the suspension culture studies involves

1. To develop a protocol for establishing cell suspension cultures of *A. aspera*.
2. To study the growth of cells in suspension culture.
3. To extract, identify and quantify 20-hydroxyecdysone from suspension cultures of *A. aspera*.
4. To study the effect of methyl jasmonate as elicitor on the production of 20-hydroxyecdysone from suspension cultures of *A. aspera*.
5. To study the effect of cholesterol and 7-dehydrocholesterol as precursors in the production of 20-hydroxyecdysone from suspension cultures of *A. aspera*.

3.3 **REVIEW OF LITERATURE**

Plant suspension cell culture is considered as free cells or small groups of cells obtained from the callus cultured in a liquid medium, which can synthesise secondary metabolites for commercial and pharmaceutical applications (Moscatiello et al. 2013). There are various advantages of using cell culture method over conventional cultivation of whole plants (i) constant supply of fresh material and valuable compounds can be synthesised in controlled conditions independent of climatic changes (ii) cultured cells remain free from microbes and insects (iii) the cells of several plants can be effortlessly multiplied to harvest specific metabolites and the production system is highly sustainable (iv) an automatic control of cell growth as well as a rational regulation of metabolite processes would decrease labour costs and also improve productivity (Vanisree et al. 2004). Likewise, plant cell suspension culture technology is extensively used for the production of high-
value natural products with pharmaceutical applications such as paclitaxel (Taxol) an anticancer drug produced by *Taxus* species by cell culture methods (Onrubia et al. 2013). *Panax notoginseng* cell suspension cultures synthesise saponin, which is an effective inhibitor of tumour promoters (Zhang et al. 1996). *Lithospermum erythrorhizon* cell suspension cultures produce shikonin derivatives which exhibit anti-tumour, anti-inflammatory, wound-healing and antimicrobial activities (Yamamoto et al. 2000). Terpenoid indole alkaloids, comprising catharanthine, vindoline, ajmalicine, bisindoles vinblastine and vincristine are synthesised by *Catharanthus roseus* cells suspension cultures (Zhao et al. 2001).

Culture conditions play a vital role in the quality and quantity of the secondary metabolites obtained through plant cell suspension cultures and optimization of the culture condition is essential for enhancing the synthesis of the desired metabolite. The culture media are augmented with the required quantity of sucrose as well as plant growth regulators suitable for cell suspension cultures (Han and Zhong, 2003) and the variation existed between them is in the nutrient levels of carbon, nitrogen, phosphate and inorganic mineral (Gamborg et al. 1968).

Previous reports are available on plant growth regulators and their manipulation to obtain high levels of valuable natural products like paclitaxel (Pan et al. 2000), ginsenosides (Zhong et al. 1996) and resveratrol (Yue et al. 2011) etc. In *Eriobotrya japonica* suspension cultures, when cultured in the medium containing 2.5 mg L$^{-1}$ of BA, 1 mg L$^{-1}$ of NAA led to high level of total triterpene production (Ho et al. 2010). Culture temperature and light conditions also influence the cell cultures; ultraviolet (UV) and red light are normally used for enhancing the production of secondary metabolites. *Ammi majus* cell culture was carried out in the optimal temperature of 20-22 °C (Staniszewska et al. 2003) and in *Catharanthus roseus* plant cells, production
of ajmalicine was found to be optimal at 27.5 °C (Ten Hoopen et al. 2002). The synthesis of catharanthine in *Catharanthus roseus* cell suspension cultures was enhanced by UV-B (Ramani and Chelliah, 2007) and exposure of red light in *Vitis vinifera* cell suspension culture improved the productions of stilbene and anthocyanins (Tassoni et al. 2012).

Cell suspension cultures can be successfully used for studying biosynthetic pathways (Lim and Bowles, 2012). They have also proven to be excellent starting materials for the protoplast isolation and to be used in a wide array of applications comprising of cell fusion and genetic manipulation (Hall, 1991).

Throughout the past decade, significant effort was made to stimulate the formation and accumulation of secondary metabolites by plant cell cultures (Ravishankar and Venkataraman, 1993; Dixon, 1999; Ravishankar and Ramachandra, 2000). Elicitation had opened up a novel area of research, which possibly increased economic benefits for pharmaceutical industry. Elicitors are signals triggering the formation of secondary metabolites. Jasmonic acid and its methyl ester (methyl jasmonate) are testified to play an essential role in a signal transduction process that controls defence genes in plants (Farmer and Ryan, 1990). Signalling molecules like jasmonic acid, methyl jasmonate and salicylic acid play a crucial role in eliciting biological responses and also have been widely used for the increased accumulation of secondary metabolites in cell and organ cultures (Yu et al. 2002; Kim et al. 2004). Methyl jasmonate, induced by insect damage, triggered the production phytoecdysteroid in *Spinacia oleracea* (Schmelz et al. 1999).

Addition of exogenous precursor to culture medium is testified to enhance the yield of the desired products. In *Vitex glabrata* cell suspension cultures precursor feeding is adopted to enhance the production of
20-hydroxyecdysone (Sinlaparaya et al. 2007). Data obtained from the radiolabeled experiments established that mevalonic acid, cholesterol and acetyl-CoA are direct precursors of phytoecdysteroid biosynthesis. (Nagakari et al.1994; Adler and Grebenok, 1999).

3.4 MATERIALS AND METHODS

3.4.1 Establishment of Cell Suspension Cultures

For initiation of cell suspension cultures, calli obtained from in vitro grown leaves were used. The calli (0.5g FW) were inoculated in 250 mL flasks containing 100 mL of MS medium supplemented with 2, 4-D (1 mg L⁻¹) and NAA (1 mg L⁻¹) and maintained on a rotary shaker at 100-105 rpm in complete darkness.

3.4.2 Determination of growth curve of cell suspension cultures

To determine the growth curve of cell suspension, the cells were separated from suspension culture by filtering the cultures. The biomass was estimated by measuring the fresh (FW) and dry weight (DW). The dry weight (DW) was calculated after drying the cells at 60 °C, for 5-6 h till no change in weight was observed. Cultures were harvested in triplicate and the biomass accumulation was examined periodically after every 5 days interval for a total period of 30 days.

3.4.3 Preparation and addition of Methyl jasmonate as elicitor

Methyl jasmonate (Sigma Aldrich, Saint Louis, USA) was prepared by diluting in dimethylsulfoxide (DMSO, Merck, India) to obtain 1M stock solution and then filter-sterilized through a sterile microfilter of 0.22 µm (Himedia, India). Elicitor was aseptically added to the 15 day old culture medium in the following final concentrations of 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 mM. The cultures were maintained on a rotary shaker at 100 rpm in
complete darkness. The cells were harvested after, 3, 6 and 9 days after addition of methyl jasmonate. Control cultures were treated with DMSO alone in the above mentioned concentrations.

3.4.4 Addition of cholesterol and 7-dehydrocholesterol as the precursor

Two different precursors such as cholesterol (Sigma Aldrich, Saint Louis, USA) and 7-dehydrocholesterol (Sigma Aldrich, Saint Louis, USA) were used at different concentrations (5, 10, 50, 100 mg L\(^{-1}\)) by adding aseptically to the 15 day old culture medium. The cultures were maintained on a rotary shaker at 100 rpm in complete darkness. The cells were harvested after, 3, 6, 9 and 12 days after addition of precursor. An appropriate control culture was maintained for each experiment.

3.4.5 Extraction of 20-hydroxyecdysone from cell suspension cultures of A.aspera

For the extraction of 20-hydroxyecdysone, 0.3 g of dried powdered cells was extracted with 20 mL methanol. The extract was then evaporated to dryness and dissolved in 1 mL methanol and filtered through a nylon syringe filter of pore size 0.22 µm to remove any cellular debris and 20 µL was injected for analysis by HPLC.

The aqueous cell suspension culture medium was extracted with ethyl acetate. The ethyl acetate layer was concentrated and filtered through a syringe filter of pore size 0.22 µm and was analyzed by HPLC.

3.4.6 HPLC analysis

The cell suspension culture extract was analyzed by HPLC using conditions and procedures explained in materials and methods of section 2.4.3.
3.4.7 LC-Q-TOF analysis

LC-Q-TOF analysis was performed using conditions described in the materials and methods of section 2.4.4.

3.4.8 Quantification of 20-hydroxyecdysone from cell suspension cultures

The amount of 20-hydroxyecdysone present in the cell suspension cultures were quantified by HPLC using the procedure described in the materials and methods of section 2.4.5.

3.4.9 Statistical Analysis

All experiments were repeated three times. The data shown represent the mean ± SE for three independent experiments. One way ANOVA analysis followed by Duncan’s Test was used to compare the means. All statistical analysis was performed using SPSS Ver.18 (SPSS Inc. Chicago, IL, USA) statistical software package.

3.5 RESULTS

3.5.1 Initiation of cell suspension cultures of A.aspera

Cell suspension cultures of A.aspera were initiated by transferring 20 day old friable type I callus to liquid MS medium supplemented with combinations of 2, 4-D (1 mg L$^{-1}$) and NAA (1 mg L$^{-1}$). The callus was easily broken apart and dispersed into single cells and cell clumps. Agitation rapidly disintegrated cell clumps into single cells and small cell aggregates. The suspension culture exhibited various degrees of cell aggregation (4 - 20 cells) which may be due to the tendency of cells to be not separate after division. Freely suspended cells and cell clusters in cell suspension culture of A. aspera are shown in Fig.3.1. The combination of 2, 4-D (1 mg L$^{-1}$) and NAA (1 mg L$^{-1}$) produced maximum callus in callus culture and it also supported growth of cells in the suspension culture.
Fig. 3.1 Microscopic observation of cells and cell clusters in suspension cultures of *A. aspera* cultured on MS medium containing 2, 4-D (1 mg L\(^{-1}\)) and NAA (1 mg L\(^{-1}\)). (a) Single cells in suspension cultures. (b and c) cell aggregates in suspension cultures.

3.5.2 Growth parameters of cell suspension cultures

The growth kinetics of cell suspension culture based on fresh weight and dry weight were also studied. The cell suspension cultures showed a growth curve with an initial lag phase up to 2-3 days, followed by an exponential phase of 5-20 days where a gradual increase in the fresh weight was observed and reached the greatest value on day 20. The initial inoculum size of 0.5 g was multiplied to 3.34±0.05 g FW and 1.09 ±0.09 g DW (Fig. 3.2) respectively. Exponential phase was terminated in a decline phase characterized by no increase in fresh weight.

Fig. 3.2 Growth curve of cell suspension cultures of *A. aspera* based on fresh and dry weight.
3.5.3 Identification of 20-hydroxyecdysone using HPLC and LC-Q-TOF analysis

20-hydroxyecdysone profile in *A.aspera* cell suspension extract were analysed by HPLC with 20-hydroxyecdysone standards from Sigma-Aldrich. HPLC analysis of cells in cell suspension gave a peak with similar retention time as authentic 20-hydroxyecdysone (Fig.3.3).

![HPLC analysis of 20-hydroxyecdysone](image)

**Fig.3.3** HPLC analysis of 20-hydroxyecdysone from cell suspension cultures of *A.aspera*. (a) 20 hydroxyecdysone reference standard (b) extract of cells in suspension culture of *A.aspera*. 
Mass spectrum of the cell extract was identical to that of the standard solution. The results clearly revealed peaks at \( m/z \) 479 (M-H)\(^-\) and exhibit an adduct ion at \( m/z \) 525 (M+HCOO)\(^-\) (Fig.3.4) in the negative ion mode obtained as a result of adding 0.1% formic acid in the mobile phase. In addition, fragment ions at \( m/z \) 319, 301, 283, 159 and 83 (Fig.3.5) were also observed in the ESI-MS/MS spectrum. The mass spectrum of the standard solution (20-hydroxyecdysone) is shown in the Fig.2.4a and Fig.2.5a of chapter two. The HPLC and LC-Q-TOF analysis clearly indicated the presence of 20-hydroxyecdysone in the extract of cells in suspension culture.

**Fig.3.4** LC-Q-TOF analysis of 20-hydroxyecdysone from cell suspension cultures of *A. aspera* (\( m/z \) 479 [M-H]\(^-\) and \( m/z \) 525 [M+HCOO]\(^-\)).

**Fig.3.5** LC-Q-TOF MS/MS (Negative mode) of the mass \( m/z \) 479(M-H)\(^-\), and its fragment.
3.5.4 Quantification of 20-hydroxyecdysone from cell suspension cultures of *A. aspera*

Data concerning the production of 20-hydroxyecdysone in cell suspension culture of *A. aspera* are shown in Fig.3.6. After 20 days of culture, the biomass accumulated was about 3.34±0.05g FW/flask and the concentration of 20-hydroxyecdysone increased progressively with time up to 20 days. The greatest value of 20-hydroxyecdysone, 0.24 mg g$^{-1}$ DW was attained on day 20, and thereafter declined rapidly (Fig.3.6). Analysis of the liquid suspension culture medium does not show the presence of 20-hydroxyecdysone.

![Fig.3.6](image)

**Fig.3.6** Time course of 20-hydroxyecdysone production in cell suspension culture of *A. aspera*. Data represent mean ± SE of three replicates and the means with the same letter are not significantly different according to the Duncan’s multiple range test ($p\leq0.05$).
3.5.5 Impact of elicitor (methyl jasmonate) addition on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*

Addition of methyl jasmonate as elicitor triggered the synthesis of 20-hydroxyecdysone in cell suspension cultures of *A. aspera*. At all concentrations tested methyl jasmonate improved the production of 20-hydroxyecdysone in *A. aspera* cell suspension cultures, and the maximum accumulation was observed at 6 days after elicitation. Methyl jasmonate at 0.6 mM gave the highest concentration of 20-hydroxyecdysone (0.352 mg g\(^{-1}\) DW) after 6 day elicitation (Fig. 3.7), which was higher as compared to the control cells.

**Fig. 3.7** Effect of methyl jasmonate on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*. Methyl jasmonate incubation period 3, 6 and 9 days are treated as different groups and data represents mean ± SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan’s multiple range test (\(p \leq 0.05\)).
3.5.6 Impact of cholesterol treatments on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*

Results indicated that there was no significant variation exhibited between the growths of cells under different treatments of precursor when compared with that of the control. Fig. 3.8 shows the effect of cholesterol on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*. The addition of cholesterol improved the production of 20-hydroxyecdysone. The maximum amount of 20-hydroxyecdysone was found to be 0.31 mg g\(^{-1}\) DW after 6 days using 10 mg L\(^{-1}\) of cholesterol.

**Fig.3.8** Effect of cholesterol on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*. Cholesterol incubation period 3, 6, 9 and 12 days are treated as different groups and data represents mean ± SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan’s multiple range test (*p*≤0.05).
3.5.7 Impact of 7-dehydrocholesterol treatments on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*

As shown in the Fig. 3.9 effect of 7-dehydrocholesterol on 20-hydroxyecdysone production in *A. aspera* cell suspension culture was also studied. The supplementation of 7-dehydrocholesterol does not affect the cell growth while the maximum amount of 20-hydroxyecdysone was found to be 0.28 mg g\(^{-1}\) DW after 6 days using 10 mg L\(^{-1}\) of 7-dehydrocholesterol.

![Fig. 3.9](image_url)

**Fig. 3.9** Effect of 7-dehydrocholesterol on 20-hydroxyecdysone production in cell suspension cultures of *A. aspera*. 7-dehydrocholesterol incubation period 3, 6, 9 and 12 days are treated as different groups and data represents mean ± SE. The means with the same letter for the same parameter do not differ significantly according to the Duncan’s multiple range test (*p*≤0.05).
3.6 DISCUSSION

In the present study, plant cell suspension cultures from *A. aspera* were initiated, maintained and analysed for the production of the phytoecdysteroid 20-hydroxyecdysone. The easiness in the establishment of suspension culture from callus tissue was influenced by the friability of the callus tissue. To obtain a fine suspension culture, yellow friable type I callus obtained from leaf tissue in MS medium supplemented with 2, 4-D (1 mg L\(^{-1}\)) and NAA (1 mg L\(^{-1}\)) were used. Literature states that there was an increase in the degree of friability of the callus tissue when they were sustained on a semisolid medium for two to three passages (Bhojwani and Razdan, 1990).

A number of growth and production media are adopted for cell suspension cultures, like Murashige and Skoog (MS) liquid medium, Gamborg (B5) liquid medium, Linsmaier and Skoog (LS) liquid medium, N6 liquid medium and other improved liquid media were also used according to the growth kinetics of plant cells (Schenk and Hildebrandt, 1972; Coste et al. 2011). In this experiment, full strength MS medium was selected for initiating cell suspension cultures from *A. aspera*. Throughout the incubation period, a gradual increase in the biomass of suspension cultures was observed due to cell division and cell enlargement. This is in agreement with *Gymnema sylvestre* cell suspension cultures where, full-strength MS medium was appropriate for biomass accumulation and gymnemic acid production (Nagella et al. 2011).

The growth pattern of cell suspension culture was analysed by measuring the fresh weight and dry weight. Starting from the day zero, when the cells were transferred into the liquid MS medium to the third day, the cells were in the lag phase which is followed by an exponential phase of 5-20 days. From 20-30 days, declining phase started where there was a gradual decrease in the fresh and dry weight. The decline in cell biomass may be due
to the consumption of the nutrients and deficiency of oxygen in the medium leading to the high percentage of cell death.

The profile of secondary metabolites in suspension culture is assumed to be similar to that of the intact plant. However, these profiles might differ and certain metabolites might be absent, whereas new metabolites not present in intact plants can appear. Our analysis of 20-hydroxyecdysone profile in cell suspension cultures of *A. aspera* specified the capability of the dedifferentiated cells to synthesise the phytoecdysteroid 20-hydroxyecdysone which is produced by the intact plant. Evidence from HPLC and mass spectra obtained from LC-Q-TOF analysis of the cell suspension of *A. aspera*, when compared with that of the authentic standard confirmed the presence of 20-hydroxyecdysone. The cells cultured in MS medium showed highest biomass and 20-hydroxyecdysone concentration (0.24 mg g\(^{-1}\) DW) at 20\(^{th}\) day of culture. This was the first report on establishing and characterising of 20-hydroxyecdysone from cell cultures of *A. aspera*. Literature data on 20-hydroxyecdysone accumulation by *Vitex glabrata* cultures showed that the amount of 20E was found to be 0.33 mg g\(^{-1}\) DW in cell suspension cultures (Sinlaparaya et al. 2007). Cheng et al. (2008) reported increased concentration (6.9 mg g\(^{-1}\) DW) of 20-hydroxyecdysone to be present in 10 to 15-month-old *Ajuga turkestanica* cell suspension cultures (Cheng et al. 2008).

The main hurdle for commercial application of cell cultures in 20-hydroxyecdysone production is the lower yields of the secondary metabolite. The yield enhancement strategies like optimization of medium, precursor feeding as well as elicitor addition is necessary to enhance 20-hydroxyecdysone production in cell cultures (Sinlaparaya et al. 2007; Cheng et al. 2008; Thanonkeo et al. 2011).

Methyl jasmonate is generally applied as an elicitor in plant cell culture (Mirjalili and Linden, 1996; Ketchum et al. 1999). The use of methyl
jasmonate and other jasmonic acid derivatives as elicitors has been fruitfully adopted in many plant species for the improvement of a particular secondary metabolite. In the current study, the content of 20-hydroxyecdysone was increased when cell suspension cultures were elicited with 0.6 mM methyl jasmonate for 6-days. It coincides with the previous report that, in *Ajuga turkestanica* cell suspension cultures, the amount of 20-hydroxyecdysone increased by the addition of 125 or 250 µM methyl jasmonate, compared to that of unelicited cultures (Cheng et al. 2008).

Addition of precursors cholesterol and 7-dehydrocholesterol (10mg L\(^{-1}\)) to the *A. aspera* cell suspension cultures enhanced the production of 20-hydroxyecdysone, compared to the control cultures. Thanonkeo et al. (2011) reported that, in *Vitex glabrata* cell cultures supplementation of cholesterol at lower concentration (5 mg L\(^{-1}\)), yielded the 1.11-fold higher amount of 20-hydroxyecdysone than that of the control cells. In *Ajuga lobata* D. Don suspension cultures, medium augmented with mevalonic (MVA), \(\alpha\)-Pinene, and nitric oxide (NO) enhanced cell growth and 20-hydroxyecdysone accumulation (Qian et al. 2016). Conversely, several reports also indicate that addition of precursor such as cholesterol, sodium acetate or mevalonic acid at 50, 100 or 150 mg L\(^{-1}\) to culture medium did not enhance 20-hydroxyecdysone accumulation in *Ajuga turkestanica* cell cultures (Cheng et al. 2008).

In conclusion, the study displayed that the cell suspension culture of *A. aspera* retained the biosynthetic potential to produce 20-hydroxyecdysone. The elicitor methyl jasmonate and precursors cholesterol and 7-dehydrocholesterol enhanced the production of 20-hydroxyecdysone in cell suspension cultures. However, further research concerning the medium optimisation and culture conditions is essential for improving the production of 20-hydroxyecdysone in cell suspension cultures of *A. aspera*. 