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Rosmarinic acid production of *Ehretia asperula* Zollinger & Moritzi cell suspension cultures: effects of cell aggregate size, glucose, and chitosan

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Abstract

Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxy phenyl lactic acid with anti-oxidant, anti-bacterial, anti-viral, and anti-cancer properties. It has been found in some plants of the Boraginaceae and Lamiaceae families. *Ehretia asperula* Zollinger & Moritzi is a medicinal plant, widely distributed in some countries such as China, Vietnam, Myanmar, and Thailand. Cell suspensions were obtained by transferring 1 g of friable callus into 20 mL of B5 liquid medium supplemented with 0.4 mg/L NAA and 0.1 mg/L BA, on orbital shaker at 90 rpm in the dark. The subculture was carried out after each of 21 days along with removing brown cell aggregates. To investigate the effects of cell aggregation, glucose, and chitosan on cell biomass and RA biosynthesis, the yellow cell suspensions were transferred to a new liquid medium. The results showed that non-sieved cell aggregates produced the highest biomass and RA content of *E. asperula* Zollinger & Moritzi cell suspension cultures. Glucose concentrations had direct effects on RA production. An addition of 45 mg/L glucose showed a significant (1.198 fold) increase in RA yield compared with control (30 mg/L) and other concentrations. On the other hand, the optimum concentration of chitosan for enhancing RA content was found at 50 mg/L after 48 hours of treatment, with an RA concentration 1.17 times greater than the control. These findings suggested that the using of cell clusters which size more than 0.125 mm, the addition of appropriate concentrations of sugar and chitosan could stimulate RA biosynthesis of *E. asperula* Zollinger & Moritzi cell suspensions.

Keywords: Cell suspension cultures, Chitosan, *Ehretia asperula* Zollinger & Moritzi, Glucose, Rosmarinic acid.
Abbreviations: 2,4-D_2,4-dichlorophenoxyacetic acid, BA_benzyl adenine, B5_Gamborg medium, FW_Fresh cell weight, KIN_kinetin, MS_Murashige and Skoog medium, NAA_naphthalene acetic acid, RA_rosmarinic acid.

Introduction

E. asperula Zollinger & Moritzi is used as a traditional medicine for the treatment of liver detoxification, ulcers, tumors, inflammation, and enhancing the body's resistance in Vietnam (Thuy et al., 2007). In Vietnam, leaves of *E. asperula* Zollinger & Moritzi have been used for tea as a healthy drink. In 1997, the results of research by Kuo and Kuo on testing the activity of ethanol bark extracts showed that this extract could be resistant to liver cancer cells, nose cancer, colon cancer, and HIV in H9 lymphocytes. In 1999, Trung et al. discovered four main compounds in this tree, namely flavonoid, quinone, triterpenoid saponin, and pyrocatechin, limiting the growth of melanoma. In 2006, Ly et al. determined that the main components in the extract of *E. asperula* Zollinger & Moritzi leaves were RA and lithospermic B acid. Next, Ly (2016) presented a procedure for extracting RA from the 50% ethanol extract of its dried leaves.

RA is a highly bioactive polyphenol compound, found in significant amounts of the Boraginaceae and Lamiaceae families (Bhatt et al., 2013). It has anti-oxidant, anti-HIV-1, anti-bacterial, and anti-cancer activities. Therefore, today, RA is extracted from plants widely used in food, cosmetic, and pharmaceutical industries (Shekarchi et al., 2012; Nadeem et al., 2019).

In recent years, the trend of multiplying plant biomass in the laboratory on a large scale is becoming a solution to gradually replace traditional farming methods. Plant cell cultures represent a potential method for obtaining valuable secondary compounds. For RA, studies have been conducted to get this compound from cell suspension cultures of many plants such as *Rosmarinus officinalis* (Kuhlmann and Röhl, 2006), *Agastache rugosa (*Park et al., 2016), *Dracocephalum moldavica* L. (Weremczuk-Jeżyna et al., 2017), *Satureja khuzistanica* (Sahraroo et al., 2018). In such a culture system, the nutrients play an important role in the initiation and production of secondary compounds by controlling enzyme activities and gene expression.

Cell aggregate size plays an important role in the production of high biomass or bioactive compounds in cell suspension cultures (Haida et al., 2019). When the plant is stress, the production of secondary compounds may increase because the growth is normally inhibited and the primary carbon source is allocated mainly to the biosynthetic activity of secondary metabolites (Akula et al., 2011). Increased

sucrose concentrations in the medium lead to RA production were higher in *Ocimum sanctum* (Hakkim et al., 2011), *Salvia leriifolia* Benth. (Modarres et al., 2018). In addition, the use of elicitors is also highly effective to enhance secondary compounds in plant cell cultures. The effects of chitosan on the accumulation of RA have been described in cell cultures specifically with *Morinda citrifolia* L. (Baque et al., 2012), *Melissa officinalis* L. (Shabini et al., 2019).

The present study is focused on investigating the effects of cell aggregate, glucose, and chitosan on the production of RA in *E. asperula* Zollinger & Moritzi cell suspension cultures.

Results and discussion

Effects of cell aggregate on cell biomass and RA production

Cell aggregates can influence cell proliferation as well as the biosynthesis of bioactive compounds in suspension cultures. In this study, cell aggregates were separated into two fractions: <0.125 mm and >0.125 mm, obtained by sieving the cells through the 0.125 mm stainless steel mesh (Fig. 2). After 4 weeks of culture, the biomass of *E. asperula* Zollinger & Moritzi cell suspensions rose as the increasing of cell aggregate cell (Table 1). In which, non-sieved cells (control) produced the highest biomass (120.533 mg/L), followed by cell aggregates with sizes >0.125 mm (105.922 mg/L) and cell aggregates with sizes <0.125 mm produced the least biomass (80.162 mg/L).

The plant cell suspensions are created by undifferentiated meristematic cells. After division, they may not separate and form cell aggregates of varied sizes and shapes. These clusters can contain up to 100 millimeter-sized cells. Cell-cell connection promotes cell aggregation by allowing intracellular communication and transport of intermediary chemicals for the biosynthesis of secondary metabolites (Capataz-Tafur et al., 2011). Therefore, cell suspension cultures with a variety of cell aggregates can promote better cell growth.

Similarly, a study on the effects of cell aggregate size on biomass in *Beta vulgaris* cell suspension cultures was conducted by Capataz-Tafur et al. (2011). The cell aggregates were divided into two fractions: <500 µm and >500 µm. The results and photomicrographs of aggregates showed that *B. vulgaris* cell suspensions tended to form larger aggregates during cell growth. In *Ficus deltoidea* var. *kunstleri* cell suspension cultures, the fractions were classified according to their sizes (500-750 µm, 250-500 µm, and <250 µm). Cell aggregates with sizes >500 µm produced higher biomass (Haida et al., 2019).

Table 1 also showed that cell aggregates affected intracellular RA biosynthesis. The highest RA content was found in non-sieved cells with 0.770 mg/g FW. On the other hand, the lowest RA production in cell suspensions was 0.606 mg/g FW for cell aggregates with sizes <0.125 mm.

RA was not only produced in cells but also secreted into the culture medium in significant concentrations. The results in Table 2 showed that the highest RA yield (179.126 mg/L) was in non-sieved cell aggregates compared with cell aggregates with sizes >0.125 mm and <0.125 mm (158.758 mg/L and 110.682 mg/L, respectively).

Many problems with cell aggregation have also been reported, such as insufficient mass transfer, hydrodynamic stress, and high viscosity in the high-density of cell cultures. However, cell aggregates can increase the production of higher secondary compounds because of their differentiation and different growth conditions (Miao et al., 2013). This has been observed in several plant cell culture systems, such as *B. vulgaris* L. (Capataz-Tafur et al., 2011) and *F. deltoidea* var. *kunstleri* (Haida et al., 2019). On the contrary, the size of the aggregate cells did not significantly affect the wilforgine and wilforine content in cell suspension cultures of *Tripterygium wilfordii* Hook. f. (Miao et al., 2013). Thus, the sizes of cell aggregates in suspension cultures may also be one of the key factors in producing biomass and bioactive compounds.

In the study, cell aggregates after 21 days without passing through the sieve were transferred to a new medium with an application of a two-stage culture system.

Effects of glucose on cell biomass and RA production

Carbon source is one of the most important factors added to the culture medium for the *in vitro* plant cells growth and production of secondary metabolites because of its roles as a carbon supplier and osmotic agent (Sahraroo et al., 2018). Biosynthesis of RA *in vitro* plant cell cultures is significantly affected by the carbohydrate content supplemented to the nutrient medium. However, the concentration of carbohydrates provided in the medium for the maximum yield of RA was different for different plant cells (Hakkim et al., 2011). For example, for *Lavandula vera* MM cell suspension cultures, 7% of sucrose in the nutrient medium ensured a steady growth of cell suspension cultures and increased the yield of RA (Ilieva and Pavlov, 1997), for *Zataria multiflora* Boiss., the best RA production/growth ratio was recorded in callus cultures supplemented with 7.5% of glucose under light condition (Françoise et al., 2007), while for *S. leriifolia* Benth., cell cultures with 4% of sucrose produced the highest biomass but the highest concentration of RA was observed in media containing 5% of sucrose (Modarres et al., 2018). In this study, the biomass and RA production of *E. asperula* Zollinger & Moritzi cell suspension cultures in a B5 medium supplemented with different concentrations of glucose (30-75 g/L) was observed. In Table 3, the results showed that when the concentration of sugar in the nutrient medium was increased, cell biomass decreased gradually. In which, the treatment supplemented with 30 g/L glucose had the highest biomass (126.889 mg/L) and the lowest biomass was cultured in a medium containing 75 g/L glucose (112.600 mg/L). This could be due to the high osmolarity which would lead to metabolic modifications in strongly hypertonic conditions (Hakkim et al., 2011). Thus, elevated glucose concentrations may cause cell dehydration and ultimately reduced cell proliferation.

RA content in the cells was affected by the concentration of glucose in the medium. The results in Table 3 also showed that the highest amount of RA was observed in the treatment containing 45 g/L glucose (0.881 mg/g FW), 1.449 fold higher than control (0.608 mg/g FW).

As shown in Table 4, RA yields of *E. asperula* Zollinger & Moritzi cell suspension cultures in the treatments with glucose higher than 30 g/L were increased. In which, the treatment containing 45 g/L glucose produced RA with the highest concentration up to 197.609 mg/L, 1.198 fold times more than control. RA was significantly present both in cells and the culture medium.

In *S. khuzistanica* cell suspension cultures, a high RA yield (4350 mg/L) was achieved when 45 or 60 g/L sucrose was added to the liquid medium at the end of cultivation (day 21). Although sucrose concentration influenced RA synthesis in different plants, the optimal concentration of this

Table 1. Effects of cell aggregates on the biomass and RA production of *E. asperula* Zollinger & Moritzi cell suspension cultures.

Cell aggregate size (mm)	FW (mg/L)	RA content in biomass (mg/g FW)
Non sieved	120.533 ± 7.847[b]	0.770 ± 0.005[c]
>0.125	105.922 ± 11.267[b]	0.690 ± 0.017[b]
<0.125	80.162 ± 4.561[a]	0.606 ± 0.005[a]

*Means followed by different superscript letters in the same column present significant differences (P ≤ 0.05).

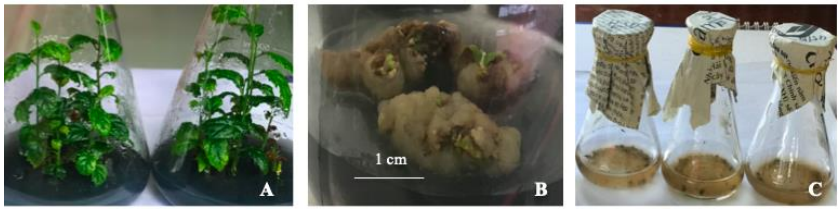

Fig 1. Twelve-week-old *in vitro*-grown plantlets of *E. asperula* Zollinger & Moritzi (A), subculturing and proliferation of callus (B), and cell suspensions in Erlenmeyer flask (C).

Table 2. Effects of cell aggregates on RA yield of *E. asperula* Zollinger & Moritzi cell suspension cultures.

Cell aggregate size (mm)	RA yield (mg/L)		
	In biomass	In liquid medium	Total
Non sieved	91.273 ± 2.423[c]	87.853 ± 2.129[b]	179.126 ± 3.884[c]
> 0.125	73.050 ± 1.796[b]	85.708 ± 2.025[b]	158.758 ± 0.238[b]
< 0.125	48.576 ± 0.447[a]	62.106 ± 0.805[a]	110.682 ± 1.212[a]

*Means followed by different superscript letters in the same column present significant differences (P ≤ 0.05).

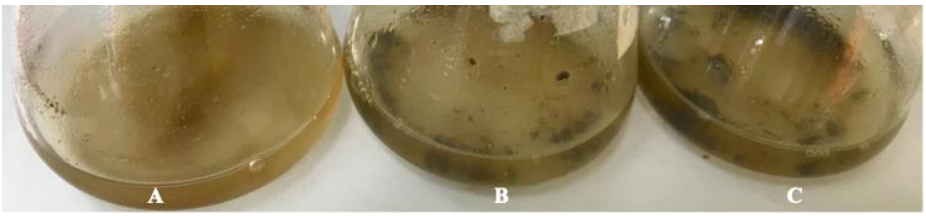

Fig 2. Cell aggregates of *E. asperula* Zollinger & Moritzi with fractions: <0.125 mm (A), >0.125 mm (B), and non-sieved (C).

Table 3. Effects of glucose on the biomass and RA production of *E. asperula* Zollinger & Moritzi cell suspension cultures.

Glucose (g/L)	FW (mg/L)	RA content in biomass (mg/g FW)
30 (control)	126.889 ± 5.906[c]	0.608 ± 0.037[a]
45	118.167 ± 2.067[ab]	0.881 ± 0.011[c]
60	119.456 ± 5.557[ab]	0.855 ± 0.005[c]
75	112.600 ± 9.702[a]	0.770 ± 0.047[b]

*Means followed by different superscript letters in the same column present significant differences (P ≤ 0.05).

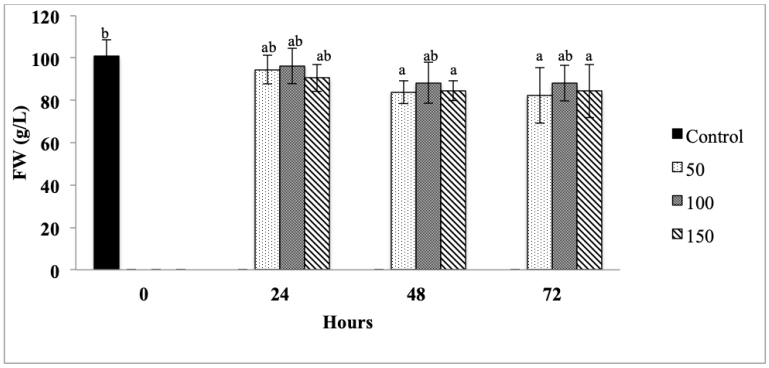

Fig 3. Effects of chitosan on the biomass of *E. asperula* Zollinger & Moritzi cell suspension cultures after 0, 24, 48, and 72 hours.

Table 4. Effects of glucose on RA yield of *E. asperula* Zollinger & Moritzi cell suspension cultures.

Glucose (g/L)	RA yield (mg/L)		
	In biomass	Liquid medium	Total
30 (control)	77.096 ± 4.758^{a}	87.853 ± 1.394^{b}	164.949 ± 6.115^{a}
45	104.124 ± 1.318^{c}	93.486 ± 0.805^{c}	197.609 ± 1.544^{c}
60	102.184 ± 0.577^{c}	80.076 ± 2.587^{a}	182.259 ± 2.561^{b}
75	86.715 ± 5.336^{b}	96.436 ± 2.587^{c}	183.152 ± 5.025^{b}

*Means followed by different superscript letters in the same column present significant differences (P ≤ 0.05).

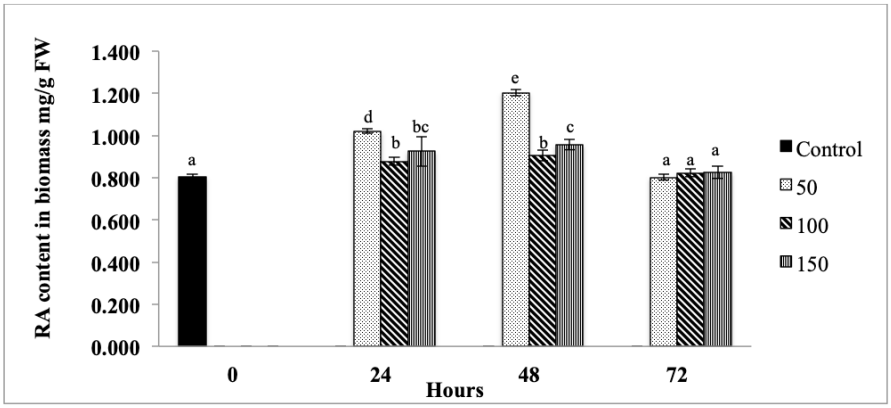

Fig 4. Effects of chitosan on RA production of *E. asperula* Zollinger & Moritzi biomass after 0, 24, 48, and 72 hours.

Table 5. Effects of chitosan on RA yield of *E. asperula* Zollinger & Moritzi cell suspension cultures after 24, 48, and 72 hours.

Chitosan (mg/L)/Harvesting time (hours)	RA yield (mg/L)		
	In biomass	Liquid medium	Total
0/0 (Control)	81.043 ± 1.487^{cd}	71.225 ± 2.323^{efg}	152.268 ± 3.759^{de}
50/24	93.141 ± 1.017^{e}	73.639 ± 3.046^{g}	166.780 ± 3.754^{f}
100/24	84.409 ± 1.757^{d}	68.543 ± 2.129^{def}	152.951 ± 3.636^{e}
150/24	83.677 ± 6.366^{cd}	65.592 ± 3.716^{cde}	149.269 ± 6.420^{cde}
50/48	100.986 ± 1.301^{f}	77.125 ± 5.931^{fg}	178.112 ± 7.162^{g}
100/48	79.887 ± 2.186^{c}	64.788 ± 2.323^{cd}	144.675 ± 4.195^{bcd}
150/48	80.838 ± 2.040^{cd}	60.765 ± 1.858^{bc}	141.603 ± 2.759^{bc}
50/72	66.029 ± 1.051^{a}	71.761 ± 5.632^{fg}	137.790 ± 5.531^{b}
100/72	72.471 ± 1.609^{b}	54.596 ± 2.587^{a}	127.066 ± 2.225^{a}
150/72	69.580 ± 2.386^{ab}	56.473 ± 2.787^{ab}	126.053 ± 4.688^{a}

*Means followed by different superscript letters in the same column present significant differences (P ≤ 0.05).

carbohydrate source varied among species and should be optimized by experimenting with different concentrations (Sahraroo et al., 2018).

Therefore, a glucose concentration of 45 g/L was added to the medium at the second stage of *E. asperula* Zollinger & Moritzi cell suspension cultures

Effects of chitosan on cell biomass and RA production

Elicitors are defined as molecules that induce defense or stress responses in plants. The exogenous elicitors are useful for studying plant responses to potential microbe attacks as well as for enhanced secondary metabolite production in cell cultures (Hakkim et al., 2011).

To determine the optimum processing time of *E. asperula* Zollinger & Moritzi cell suspensions with chitosan and its concentration to enhance RA biosynthesis as well as reduce the inhibitory effects of elicitors on the cell proliferation, the cells were exposed to chitosan at different concentrations (0, 50, 100, and 150 mg/L) on day 28 and harvested after 24, 48, and 72 hours of exposure. The results showed that when processing time of the cells with different concentrations of chitosan increased, the biomass decreased (Fig. 3). In contrast, RA accumulation increased significantly in all concentrations of chitosan compared with control (without chitosan), especially after 48 hours of culture. In which, the RA content in biomass was the highest when treated with 50 mg/L chitosan at 48 hours after elicitation (1.204 mg/g FW), 1.498 fold higher than control (Fig. 4).

According to Vanda et al. (2019), treatment of *M. officinalis* L. shoots by chitosan led to a considerable induction of phenylalanine ammonia-lyase, catalase, guaiacol peroxidase and lipoxygenase activities after 2 weeks. Also, the expression of *PAL1*, *TAT*, and *RAS* genes and production of total phenolic compounds and RA increased in all concentrations (50, 100, and 150 mg/L) of chitosan compared with control.

Time of elicitation with chitosan in cell suspensions also significantly affected the production of RA. In which, all of the treatments supplemented with chitosan and after 48 hours of elicitation had a noticeably higher yield of RA than control. However, the treatments with chitosan all had a lower weight than control, the yields of RA in some treatments with chitosan 50, 100, and 150 mg/L at 72 hours were lower than control. In which, the treatment supplemented with 50 mg/L chitosan and after 48 hours had the highest RA yield of 178.112 mg/L, 1.17 fold higher than

control (Table 5). Similarly, the effects of chitosan on the increase in biomass and metabolite compounds in adventitious root cultures of *M. citrifolia* L. were studied by Baque et al. (2012). The optimum contact period of roots to the elicitor was observed when adventitious root cultures were treated with 0.2 mg/mL chitosan on day 28 and harvest after 2 days of elicitation. Although root proliferation slightly decreased, the yields of anthraquinones, phenolics, and flavonoids were enhanced by 45%, 8%, and 12%, respectively compared with control.

Materials and methods

Callus induction and cell suspension cultures

Callus was induced from leaf explants (0.5 cm x 1 cm) of twelve-week-old *in vitro*-grown plantlets of *E. asperula* Zollinger & Moritzi (Fig. 1.A). The explants were cultured on B5 medium (Gamborg et al., 1968) supplemented with 30 g/L glucose, 7.5 g/L agar, 0.5 g/L activated coal, 0.4 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg/L benzyl adenine (BA), pH 5.8. The cultures were incubated in the dark at 25°C. The callus was subcultured after 5 weeks until it became friable (Fig. 1.B).

Cell suspension cultures were established by transferring 1 g of friable callus into 100 mL Erlenmeyer flasks containing 20 mL of B5 fresh liquid medium supplemented with 0.4 mg/L NAA and 0.1 mg/L BA (Fig. 1.C). The cultures were rotated at 90 rpm on an orbital shaker in darkness at 25°C. After 21 days, the brown cell clumps were removed. The yellow cell suspensions, consisting of single cells and small clusters, were transferred to a fresh liquid medium to investigate the influence of several factors on the proliferation and biosynthesis of RA.

Effects of cell aggregate

The 21-day-old cell suspensions cultures were sieved using a stainless steel mesh of 0.125 mm. Cell suspension fractions were classified according to their sizes: <0.125 mm, >0.125 mm, and control (non-sieved cell). Each culture vessel contained 20 mL B5 liquid medium and 1 g of cells. The cultures were rotated at 90 rpm on an orbital shaker under total darkness at 25°C for 4 weeks. Cell suspension cultures with the best aggregate were used in all subsequence experiments.

Effects of glucose

The 21-day-old cells (1 g) were transferred to 100 mL Erlenmeyer flasks containing 20 mL of fresh liquid B5 medium supplemented with different concentrations of glucose (30, 45, 60, and 75 mg/L). The cultures were rotated at 90 rpm on an orbital shaker in darkness at 25°C for 4 weeks.

Effects of chitosan

The concentration of chitosan (Glentham-UK) and processing time would be studied. Chitosan solution was prepared in aqueous acidic solution according to Vanda et al. (2019). After 4 weeks of culture, chitosan (0, 50, 100, and 150 mg/L) was added to the medium and harvested after 24, 48, and 72 hours of elicitation. The cultures were rotated at 90 rpm on an orbital shaker in dark condition at 25°C.

Growth measurement

The growth of *E. asperula* Zollinger & Moritzi was measured with fresh cell weight (FW) as well as the color of cell suspension cultures. 10 mL of cell suspension was centrifuged in a 15 mL tube for 20 minutes at 5500 rpm for the biomass.

Extraction of RA

The fresh biomass was soaked with a 50% ethanol solvent at the rate of 1:12 (w/v), then sealed and placed on a magnetic stirrer at 200 rpm, 70^0C for 3 hours. The extract was passed through filter paper retaining the clear solution to analyze RA as described by Ly (2016).

Determination of RA

RA content was analyzed following the methods described by Ozturk et al. (2010). 200 mL of extract solutions were added to a 200 µL zirconium oxide chloride solution (0.5 M) and 4.6 mL ethanol. After 5 min, the absorbance of the reaction mixture was measured at 362 nm using a spectrophotometer with RA (Sigma-Aldrich) at the concentration range of 2 – 40 µM as a standard.

Statistical analysis

The data were statistically processed using Statgraphics Centurion XV software with a significant difference of a 5% level (one-way ANOVA followed by LSD test). All experimental data shows the mean and standard deviation of triplicate experiments.

Conclusion

In this study, different factors including cell aggregate, glucose, and chitosan were used to improve the biomass and RA content of *E. asperula* Zollinger & Moritzi cell suspension cultures. For biomass production, non-sieved cells and 45 g/L glucose were considered the most appropriate. In the period of RA biosynthesis, the medium supplemented with 50 mg/L chitosan and a contact period of 48 hours were the optimal conditions for RA production in cell suspension cultures of *E. asperula* Zollinger & Moritzi.

The records can contribute new knowledge in the food, cosmetic, and pharmaceutical industries and meet the need for bioactive compounds in large quantities.

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Author's contribution

Pham Thi My Tram has done plant tissue cultures, extraction of RA, spectrophotometric analysis, guided and supervised by Assoc. Prof. Le Thi Thuy Tien and Assoc. Prof. Ngo Ke Suong.

Conflict of Interest

Authors declare no conflict of interest.

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