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Research Article

Enhanced accumulation of total phenolic content and rosmarinic acid in cell suspension cultures of *Ehretia asperula* using elicitors and precursors

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Abstract

Ehretia asperula, a Vietnamese endemic species known as Xạ Đen, has traditional medicinal applications for conditions such as hepatitis, liver cirrhosis, and cancer. Plant cell suspension cultures provide an alternative approach for producing secondary metabolites. This study focuses on the effects of precursors and elicitors on the accumulation of total phenolic and rosmarinic acid, one of the bioactive compounds presents in *E. asperula*. Suspension cultures were established using Gamborg (B5) medium supplemented with 1.5 mg L^{-1} naphthalene acetic acid. Precursors, including L-tyrosine and L-phenylalanine, and elicitors, such as salicylic acid and yeast extract, were added to the culture medium at different concentrations to examine their effects on total phenolic and rosmarinic acid production. The results indicated that precursors and elicitors differentially influenced the accumulation of secondary metabolites and the growth of *E. asperula* cells. Notably, L-tyrosine was more effective than L-phenylalanine in enhancing biomass and secondary metabolite production after 15 days of culture, with the highest yield observed at 0.5 mg L^{-1} L-tyrosine. Between the two elicitors tested, yeast extract significantly promoted the production of phenolic compounds and rosmarinic acid, with the highest levels recorded after 12 hours of treatment with 1.0 g L^{-1} yeast extract. Overall, the findings highlight the potential of *E. asperula* cell suspension cultures as a substantial source of rosmarinic acid production. The protocol developed in this study offers a promising solution to address the challenges of large-scale production and provides a potential source of raw materials for pharmaceutical, cosmetic, and functional food industries.

Keywords: L-tyrosine; L-phenylalanine; naphthalene acetic acid; salicylic acid; yeast extract

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Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid; GMP: Good Manufacturing Practice; L-tyr: L-tyrosine; L-phe: L-phenylalanine; NAA: α-naphthalene acetic acid; RosA: Rosmarinic acid; SA: Salicylic acid; TPC: Total phenolic content; YE: Yeast extract

Introduction

The *Boraginaceae*, a diverse family encompassing approximately 2000 species of herbs, shrubs, and trees, is predominantly found in Europe and Asia. Within this family, numerous plants are valued for their significant contributions to cosmetics and pharmacology (Dresler *et al*., 2017). Seventeen species from the Borage family (*Boraginaceae*) were identified to contain various bioactive compounds, including allantoin, p-hydroxybenzoic acid, rutin, hydrocaffeic acid, rosmarinic acid (RosA), chlorogenic acid, and shikonin (Dresler *et al*., 2017). This finding suggests that these Borage family plants could be a valuable source of natural health-promoting compounds.

Similarly, to many other members of the *Boraginaceae* family, *Ehretia* species are rich in secondary metabolites, including flavonoids, steroids, triterpenoids, lignans, and phenolic acids (Li *et al*., 2010). *Ehretia asperula* Zoll. et Mor. (*E. asperula*), known locally as "Xạ Đen", is an endemic species of *Ehretia* in Vietnam, found in provinces such as Hoa Binh, Tuyen Quang, and Ninh Binh (Hoang *et al*., 2021). *E. asperula* has effectively prevented acne, jaundice, hypertension, and diabetes (Nguyen *et al*., 2017). Traditionally, *E. asperula* leaves have been utilized in folk medicine to address ailments including hepatitis, liver cirrhosis, and cancer (Hoang *et al*., 2021). While some recent studies suggest anti-cancer potential for *E. asperula*, clinical trials for cancer therapy have not yet been conducted (Kim *et al*., 2019). Le *et al*. (2021) demonstrated the protective effects of *E. asperula* leaf-derived extracts against retinal cell death and elevated reactive oxygen species induced by glutamate/BSO-mediated excitotoxicity and oxidative stress. These findings suggest potential therapeutic applications of *E. asperula* in treating retinal degeneration. RosA is a major secondary metabolite of *E. asperula*, alongside other compounds such as astragalin, lithospermic acid B, and kaempferol 3-rutinoside (Tuan *et al*., 2016; Le *et al*., 2021). According to Guan *et al*. (2022), RosA exhibits various pharmacological activities, including antiviral, anti-inflammatory, antioxidant, antitumor, neuroprotective, and antidepressant effects, as evidenced by multiple *in vivo* and *in vitro* studies. Additionally, RosA is used in cosmetics because it absorbs UV radiation. It aids in stabilizing biological membranes and protecting keratinocytes from the harmful effects of UV_A and UV_B rays and reactive oxygen species, including free radicals (Sánchez-Campillo *et al*., 2009; Piao *et al*., 2024). RosA contributes to preventing skin irritation and aging by enhancing the protective function of the stratum corneum of the epidermis (Jung *et al*., 2022).

Plant biotechnology has grown substantially in recent decades, particularly in *in vitro* plant cell culture techniques. These methods offer a valuable means for preserving plant species and producing bioactive compounds without being limited by seasonal and climatic factors (Verpoorte *et al*., 1998). Scientists have achieved significant breakthroughs in utilizing plants to produce RosA through *in vitro* techniques such as callus, cell suspension, hairy roots, and shoot culture. This success extends across various plant species, including *Salvia miltiorrhiza* (Huang *et al*. 2008), *Ocimum basilicum* (Strazzer *et al*., 2011). Products obtained from *in vitro* culture systems offer several advantages over traditional methods. Their controlled environment ensures stable yield and consistent quality, independent of seasonal or environmental fluctuations. Shorter production cycles allow faster response to market demands and efficient resource utilization. Moreover, the closed-loop nature of *in vitro* systems facilitates adherence to rigorous Good Manufacturing Practice (GMP) regulations, making them ideal for producing pharmaceuticals and other sensitive products (Mamun *et al*., 2015). *In vitro* culture methods, such as adventitious root culture, hairy root culture, and cell suspension culture, can replace traditional methods requiring large cultivation areas and specific soil conditions. Traditionally, harvesting

sufficient raw materials to meet current demands can be both ineffective and unsustainable (Murthy and Praveen, 2012). Plant cell suspension culture is regarded as one of the most suitable systems for large-scale production of plant-derived secondary metabolites owing to its scalability. In addition, its strong potential for automation offers a significant advantage for mass production. Cell suspension systems can significantly improve efficiency and reduce costs associated with plant-based product manufacturing by automating critical processes within these cultures (Motolinía-Alcántara *et al*., 2021).

Nevertheless, the low yield is the disadvantage of applying *in vitro* technology and cell suspension culture in producing bioactive compounds (Skrzypczak-Pietraszek *et al*., 2018). To overcome this limitation, researchers have investigated various new and advanced techniques aimed at improving the extraction of secondary compounds. These approaches include optimizing the culture medium, selecting high-yield cell lines, utilizing precursors and elicitors, employing genetic techniques, and choosing suitable bioreactor culture systems (Marchev *et al*., 2021). One of the latest trends involves stimulating the biosynthesis of *in vitro* secondary metabolites using precursors and elicitors (Ru *et al*., 2017). Researches indicate that incorporating biotic and abiotic elicitors, such as chitosan, yeast extract (YE), methyl jasmonate (MJ), and salicylic acid (SA), into the culture medium could effectively enhance RosA production *in vitro* (Li *et al*., 2021). Elicitors can trigger plant defense responses at minimal concentrations, modulating gene expression within complex signaling networks to enhance secondary metabolite biosynthesis by activating specific metabolic pathways (Ramirez-Estrada *et al*., 2016).

Plant cell suspension cultures offer a scalable source of secondary metabolites (Isah, 2019). Scientists successfully increased the production of valuable compounds from plant cells using cell suspension cultures. In studies involving *Mentha* plants and *Coleus* cell suspension, it has been indicated that L-phenylalanine (L-phe) and L-tyrosine (L-tyr) are the exclusive direct amino acid precursors of 3,4-dihydroxy-phenyl lactic acid and caffeic acid (Ru *et al*., 2017). RosA is synthesized through the esterification of 3,4-dihydroxyphenyl lactic acid and caffeic acid (Petersen *et al*., 1993; Petersen and Simmonds, 2003). The biosynthesis of RosA comprises two pathways: one originating from phenylpropanoids and the other from tyrosine. Both pathways are integral to the phenolic acid biosynthesis pathway and rely on L-phe and L-tyr as precursors (Deng *et al*., 2020). To improve the growth and target metabolite production of plant cell suspension, researchers have explored techniques and theories at multiple dimensions, including induction, screening, and selection of high-quality cell lines, optimization of conditions and medium culture, and elicitors and precursor feeding (Marchev and Georgiev, 2020; Li *et al*., 2021).

In previous investigations (according to Tuan *et al*., 2016; Thao *et al*., 2021), RosA was identified in the leaves collected from Hoa Binh using nuclear magnetic resonance spectroscopy. In addition, friable callus was successfully induced from young stem explants, which served as the source for initiating primary cell suspensions. This primary cell suspension was subsequently used as inocula to establish and proliferate secondary suspension cultures in suitable media. Building upon these results, the present study aimed to integrate precursor feeding and elicitor supplementation strategies into suspension cultures. The present research focuses on studying the effects of varying concentrations of salicylic acid (SA) and yeast extract (YE), commonly used elicitors, as well as L-tyrosine (L-tyr) and L-phenylalanine (L-phe), well-known precursors of the phenylpropanoid pathway, on biomass accumulation, total phenolic content (TPC), and RosA production. Additionally, the effects of different exposure durations to these elicitors and precursors were evaluated to determine suitable conditions for enhancing both cell growth and secondary metabolite production in *E. asperula* suspension cultures.

Materials and Methods

Plant materials

E. asperula plants were collected from Hoa Binh Province, Vietnam, and subsequently cultivated and maintained in the greenhouse of the Plant Biotechnology and Agriculture Department, Institute of Life Sciences, VAST, in Ho Chi Minh City, Vietnam.

Chemicals and instrumentation

Plant growth regulators α-naphthalene acetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D), as well as SA, were obtained from Duchefa Biochemie (Netherlands). L-tyr, L-phe, YE, and zirconium (IV) oxide chloride octahydrate ($ZrOCl_2 \cdot 8H_2O$) were purchased from HiMedia Laboratories (India). RosA, Folin-Ciocalteu reagent, and gallic acid (3,4,5-trihydroxybenzoic acid) were supplied by Merck and Sigma-Aldrich (Merck Life Science Pty Ltd, Germany).

Experiment designs

This experiment builds upon our previous research, according to the study of Thao *et al*. (2021); therefore, the experimental methods were conducted following the established protocols described in this study. Furthermore, data on fresh weight (FW), dry weight (DW), and the timing of elicitors and precursors supplementation from Thao *et al*.'s study were utilized as reference controls for the experiments in this study.

Friable callus induction and establishment of cell suspension culture

Young stems from 1-year-old *ex vitro E. asperula* plants were harvested and sectioned into 3-5 cm segments. The collected explants were rigorously washed under running tap water for 30 minutes. Stems were surface-sterilized sequentially in 70% alcohol for 60 seconds and $HgCl_2$ 0.1% for 5 minutes. Subsequently, sterilized explants were sliced into 1.0 mm thick, 2.0 mm diameter transverse sections and cultured on Murashige and Skoog's medium (MS) (Murashige and Skoog, 1962) supplemented with 1.5 mg L^{-1} 2,4-D (Thao *et al*., 2021). Friable calli formed after 15 days of culture and were utilized as the inoculum for cell suspension culture.

The calli were transferred to B5 medium (Gamborg *et al*., 1968), containing 1.5 mg L^{-1} NAA, 30 g L^{-1} sucrose (Bien Hoa Sugar Jsc., Vietnam) (referred to as BM medium) to initiate cell suspension cultures. After that, the cell suspensions (initial fresh weight of 1.5 g) were cultured in the BM medium for biomass proliferation, following the protocol outlined by Thao *et al*. (2021). After 15 days of culture, a cell suspension was established and used as the primary material for subsequent experiments.

This treatment focused on generating plant materials for subsequent experiments rather than collecting quantitative data. Instead, callus and cell suspension were observed visually and under a microscope.

For precursor and elicitation experiments, cultures were prepared by inoculating 1.5 g of fresh weight of 15-day-old cell suspension biomass in 250 mL Erlenmeyer flasks containing 50 mL of liquid BM culture medium. Cultures were incubated in the dark on a rotary shaker at 100 rpm and 25 ± 2 °C.

Precursor feeding experiments

L-tyr and L-phe at concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 mg L^{-1} were supplemented into BM medium to evaluate the effects of precursor type and concentration on biomass production and secondary metabolite accumulation in *E. asperula* cell cultures. BM medium without precursor served as the control. Cell suspension growth kinetics were primarily assessed on days 6, 9, 12, 15, 18, and 21 of cultivation, based on FW and DW. TPC and RosA accumulation were quantified at three distinct time points, namely pre-peak (before the biomass reached its highest weight), peak (at its maximum biomass weight), and post-peak (after the peak biomass phase).

Elicitation experiments

SA at the concentrations of 5, 10, 15, 20, and 25 mg L^{-1} and YE and 1.0, 2.0, 3.0, 4.0, and 5.0 g L^{-1} for YE were supplemented into BM medium, to evaluate the effect of elicitors on the TPC and RosA accumulation as well as the biomass production of *E. asperula* cells. BM medium without elicitor was used as a control. After elicitor supplementation, biomass samples were collected at 3-day intervals, starting on day 6, throughout the 21-day culture period.

Fresh weight was calculated by filtering cell suspension through filter paper, with the initial mass of the dried filter paper measured beforehand. The cell biomass dry weight was determined following a 48-hour drying period at 50 °C.

Once the optimal type and concentration of the precursor, as well as the appropriate cultivation time for biomass accumulation, were determined, the effect of biomass harvesting time on secondary compound production was also investigated by supplementing the precursor at the point time when cell biomass reached its highest weight. The precursor and elicitor, filter-sterilized through a 0.22 µm Millipore membrane, was introduced into the culture medium. TPC and RosA accumulation were assessed at 6, 12, 24, 36 and 48 hours post-precursor addition.

Preparation of ethanol extract of E. asperula cell suspension

Ethanol extraction of *E. asperula* cell suspension biomass was conducted following the protocol outlined by Öztürk *et al*. (2010). Briefly, the biomass was subjected to four consecutive ethanol extractions (30 ml each) over 24 hours. The combined extracts were filtered and concentrated under vacuum at 50 °C. The concentrate was dried at 25 °C until constant weight was achieved and then used for TPC and RosA content quantification.

Quantification of rosmarinic acid content in the extracts of E. asperula cell suspension

Rosmarinic acid content within the ethanolic extract was quantified spectrophotometrically following the protocol established by Öztürk *et al*. (2010). A 200 µl aliquot of the ethanol extract was combined with 4.6 ml of ethanol and 200 µl of zirconium solution, subjected to vortexing for 5 minutes, and transferred to a 96-well plate. Absorbance readings were obtained at 362 nm using an ELISA Benchmark Plus Microplate Reader (Bio-Rad, USA). RosA concentrations were determined based on the standard curve with the linear regression equation $y = 0.0011x - 0.0073$ and an R^2 value of 0.9989.

Quantification of total phenolic content in extracts of E. asperula cell biomass

TPC of the *E. asperula* cell suspension ethanol extract was determined using the Folin-Ciocalteu colorimetric assay (Folin and Ciocalteu, 1927; Singleton *et al*., 1999). Briefly, 100 µL of the extract was combined with 500 µL Folin-Ciocalteu reagent, incubated for 5 minutes in the dark, and then combined with 400 µL Na_2CO_3 solution for a 60-minute incubation in the dark. Absorbance was measured at 765 nm using a 96-well plate. TPC was calculated from a gallic acid standard curve ($y = 0.0045x + 0.0404$, $R^2 = 0.9984$).

Statistical analysis

The experiments were conducted using a completely randomized design with three replicates. Data analysis was performed using one-factor and two-factor ANOVA models with Statgraphics Centurion software, and graphs were generated using Microsoft Excel 2010. A two-way ANOVA was applied to evaluate the effects of the precursor or elicitor (L-tyr, L-phe, SA, or YE at various concentrations) and culture duration on fresh biomass, dry biomass, TPC, and RosA accumulation in *E. asperula* cell suspensions. A one-way ANOVA was conducted to assess the influence of culture duration on TPC and RosA content. Significant differences among treatments were determined using Tukey's test ($p < 0.05$). The results are expressed as the mean ± standard deviation from replicated experiments.

Results

Friable callus induction and establishment of cell suspension culture

In this study, all young stem explants developed white, friable calli within 15 days of culture (Figure 1a). Cell suspensions derived from these calli were successfully established in BM medium after 15 days. Suspensions were subsequently subcultured in BM medium, and biomass was harvested after 15 days of proliferation. Figures 1b-d showed the appearance of *E. asperula* cell suspensions in BM medium on day 0 and day 15, as well as the harvested biomass. Microscopic observations on day 15 revealed numerous clusters of small, spherical cells and some elongated cell clusters (Figure 1e-h). Furthermore, the cell suspension was stained with acridine orange dye. Fluorescence microscopy images indicated that the cells were spherical and exhibited intense fluorescence. This observation is likely due to the cells being in a rapid growth phase, characterized by the synthesis of precursors and continuous division, resulting in a denser cytoplasm and nucleus and a higher affinity for the dye (Figure 1i-m).

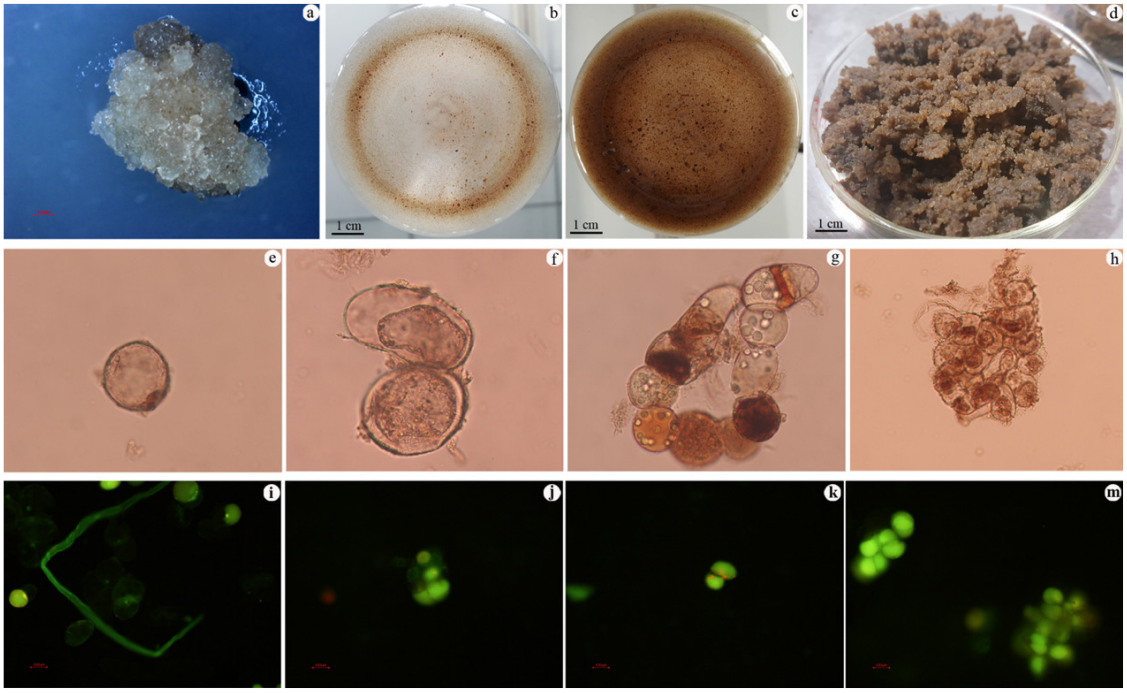


Figure 1. Callus formation, cell suspension development, biomass accumulation, and cellular morphology of *E. asperula* grown in BM medium under microscopy: (a) friable callus; (b, c) *E. asperula* cell suspension in BM medium was on the 6th and 15th day; (d) cells biomass; (e, f, g, h) optical image identifies normal and mitotic cells grown in BM medium; (i, j, k, l) fluorescence image of *E. asperula* cells

Effect of L-tyr and L-phe on the growth of E. asperula cell suspension

Two-way ANOVA was conducted to investigate the influence of precursor concentration and culture time on the growth index of *E. asperula* cells (fresh and dry weight). The results revealed a significant statistical difference in both FW and DW across experiments ($p < 0.05$) and culture periods ($p < 0.05$) (Table 1, 2).

For L-tyr, peak biomass was achieved on day 18 in the 0.5 and 1.0 mg L^{-1} L-tyr experiment, whereas it occurred earlier, on day 15, in the 1.5, 2.0, and 2.5 mg L^{-1} L-tyr experiment. Low L-tyr concentrations (0.5 and 1.0 mg L^{-1}) stimulated cell growth, resulting in higher fresh and dry weights compared to both the control and higher L-tyr concentrations (Table 1, Figure 2a, b). The 0.5 mg L^{-1} L-tyr treatment yielded the highest biomass,

with fresh and dry weights reaching 11.52 g and 0.80 g, respectively, on day 18, exceeding the control values of 9.13 g and 0.70 g on day 15 and 7.61 g and 0.61 g on day 18. However, biomass decreased to 6.96 g FW and 0.55 g DW in the 2.0 mg L^{-1} L-tyr treatment and 6.03 g FW and further declined to 0.50 g DW in the 2.5 mg L^{-1} L-tyr treatment (Table 1). Overall, supplementation with 1.5 mg L^{-1} L-tyr was found to be suitable for *E. asperula* cell suspension proliferation, with day 18 identified as the optimal time point for biomass collection.

Table 1. Effects of L-tyr on the fresh and dry weight of *E. asperula* cells from 6 to 21 days

Parameter	Culture time	L-tyrosine (mg L^{-1})						
		Control	0.5	1.0	1.5	2.0	2.5	$Means_{CT}$
Fresh weight (g)	The 6th day	2.84 ± 0.41^{v*}	3.19 ± 0.44^{uv}	3.87 ± 0.56^{s-v}	4.67 ± 0.30^{p-t}	3.01 ± 0.40^{l-v}	2.89 ± 0.46^{v}	3.41^{E}
	The 9th day	4.05 ± 0.59^{r-v}	4.39 ± 0.89^{q-u}	4.70 ± 0.34^{o-t}	5.69 ± 1.24^{l-q}	4.12 ± 0.56^{r-v}	3.61 ± 0.23^{t-v}	4.43^{D}
	The 12th day	6.07 ± 0.77^{k-o}	5.98 ± 0.59^{k-p}	6.54 ± 0.46^{j-n}	7.34 ± 1.57^{h-k}	5.19 ± 0.45^{n-s}	4.87 ± 0.46^{o-t}	6.00^{C}
	The 15th day	9.13 ± 0.50^{c-f}	8.11 ± 1.02^{e-i}	8.42 ± 0.72^{d-h}	9.17 ± 0.42^{c-e}	6.96 ± 1.29^{i-l}	6.03 ± 0.38^{k-p}	7.97^{A}
	The 18th day	7.61 ± 0.46^{g-j}	11.52 ± 0.88^{a}	10.17 ± 0.41^{a-c}	8.77 ± 0.52^{d-g}	6.64 ± 1.03^{j-m}	5.36 ± 0.44^{m-r}	8.34^{A}
	The 21st day	5.56 ± 0.48^{m-q}	10.79 ± 1.28^{ab}	9.75 ± 0.67^{b-d}	7.73 ± 0.56^{f-j}	6.41 ± 1.50^{j-n}	4.78 ± 0.46^{o-t}	7.50^{B}
	$Mean_{YE}$	5.88^{B}	7.33^{A}	7.24^{A}	7.23^{A}	5.39^{C}	4.59^{D}	
Dry weight (g)	The 6th day	0.24 ± 0.07^{p}	0.30 ± 0.04^{op}	0.33 ± 0.05^{m-p}	0.41 ± 0.03i-o	0.28 ± 0.04^{op}	0.29 ± 0.05^{op}	0.31^{D}
	The 9th day	0.31 ± 0.07^{n-p}	0.39 ± 0.07^{j-o}	0.40 ± 0.04^{i-o}	0.48 ± 0.11g-l	0.38 ± 0.05^{k-o}	0.35 ± 0.02^{l-p}	0.39^{C}
	The 12th day	0.47 ± 0.08^{g-l}	0.47 ± 0.05^{g-l}	0.51 ± 0.03^{f-k}	0.58 ± 0.13^{d-h}	0.44 ± 0.04^{h-n}	0.44 ± 0.10^{h-n}	0.49^{B}
	The 15th day	0.70 ± 0.09^{a-d}	0.61 ± 0.08^{c-f}	0.64 ± 0.07^{b-e}	0.68 ± 0.03^{a-d}	0.55 ± 0.11^{e-i}	0.50 ± 0.04^{f-l}	0.61^{A}
	The 18th day	0.61 ± 0.07^{c-g}	0.80 ± 0.07^{a}	0.74 ± 0.04^{ab}	0.65 ± 0.04^{b-e}	0.55 ± 0.12^{e-i}	0.45 ± 0.04^{h-m}	0.63^{A}
	The 21st day	0.54 ± 0.15^{e-i}	0.78 ± 0.09^{a}	0.73 ± 0.02^{a-c}	0.61 ± 0.04^{c-f}	0.53 ± 0.07^{e-j}	0.44 ± 0.04^{h-n}	0.60^{A}
	$Mean_{YE}$	0.48^{B}	0.56^{A}	0.56^{A}	0.57^{A}	0.45^{B}	0.41^{C}	

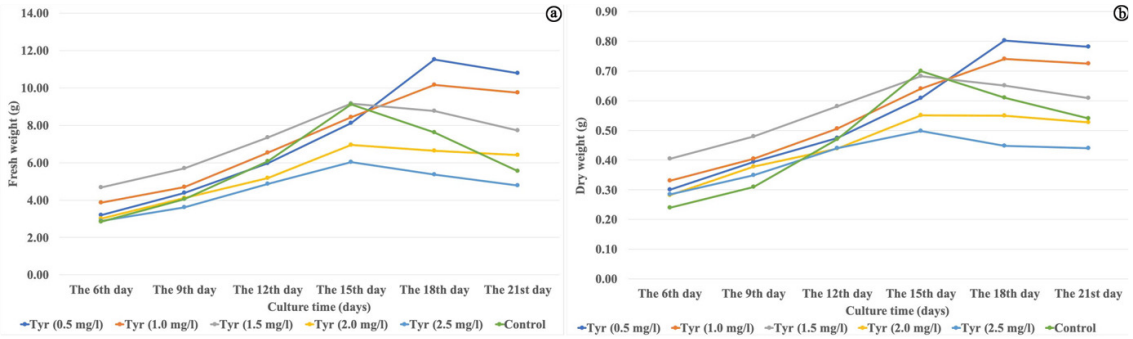
Values with different letters are significantly different according to the Tukey HSD test at $p < 0.05$. Lowercase letters indicate significant differences in the interaction between culture time and L-tyr concentration, while uppercase letters represent significant differences between treatments

Similar results were observed in the experiment with L-tyr supplementation. The growth cycle of cells in the medium supplemented with 0.5 and 1.0 mg L^{-1} L-phe was longer than in other experiments, reaching maximum biomass after 18 days of culture (Table 2). Conversely, in the subsequent three treatments, as L-phe concentration increased, cell biomass decreased, and the growth cycle shortened (Figure 2c, d). In these experiments, fresh and dry biomass weight peaked after 15 days of culture. Cells grown in the medium supplemented with 1.0 mg L^{-1} L-phe exhibited better growth than those in media with other L-phe concentrations; however, no statistically significant difference was observed compared to the control.

Table 2. Effects of L-phe on the fresh and dry weight of *E. asperula* cells from 6 to 21 days

Parameter	Culture time	L-phenylalanine (mg L^{-1})						
		Control	0.5	1.0	1.5	2.0	2.5	$Means_{CT}$
Fresh weight (g)	The 6th day	2.84 ± 0.41^{o}	3.04 ± 0.40^{no}	$3.88 \pm 1.20^{k-o}$	$4.14 \pm 0.68^{j-o}$	$3.43 \pm 0.56^{m-o}$	2.82 ± 0.44^{o}	3.36^{E}
	The 9th day	$4.05 \pm 0.59^{j-o}$	$3.98 \pm 0.51^{k-o}$	$5.02 \pm 1.12^{h-l}$	$5.30 \pm 1.12^{h-l}$	$4.41 \pm 0.80^{j-n}$	$3.80 \pm 0.22^{l-o}$	4.43^{D}
	The 12th day	$6.07 \pm 0.77^{f-i}$	$5.04 \pm 1.18^{h-l}$	$6.37 \pm 0.72^{e-h}$	$6.28 \pm 0.72^{e-h}$	$5.37 \pm 0.55^{h-k}$	$4.72 \pm 0.33^{i-m}$	5.64^{C}
	The 15th day	9.13 ± 0.50^{ab}	$7.12 \pm 1.58^{c-f}$	$8.20 \pm 1.13^{a-c}$	$8.26 \pm 0.66^{a-c}$	$7.06 \pm 1.24^{c-g}$	$6.52 \pm 0.65^{d-h}$	7.71^{A}
	The 18th day	$7.61 \pm 0.46^{c-e}$	$8.11 \pm 1.02^{a-c}$	9.26 ± 0.61^{a}	$7.87 \pm 0.79^{a-d}$	$6.32 \pm 1.31^{e-h}$	$6.04 \pm 0.68^{f-i}$	7.53^{A}
	The 21st day	$5.56 \pm 0.48^{g-j}$	$7.66 \pm 0.63^{b-e}$	$8.39 \pm 0.72^{a-c}$	$7.24 \pm 0.81^{c-f}$	$6.03 \pm 0.93^{f-i}$	$5.26 \pm 1.06^{h-l}$	6.69^{B}
	$Mean_{Phe}$	5.88^{B}	5.82^{B}	6.85^{A}	6.52^{A}	5.44^{B}	4.86^{C}	
Dry weight (g)	The 6th day	0.24 ± 0.07^{p}	0.27 ± 0.04^{op}	$0.33 \pm 0.10^{l-p}$	$0.37 \pm 0.07^{k-p}$	$0.32 \pm 0.05^{m-p}$	$0.29 \pm 0.04^{n-p}$	0.30^{D}
	The 9th day	$0.31 \pm 0.07^{m-p}$	$0.34 \pm 0.04^{l-p}$	$0.43 \pm 0.09^{g-m}$	$0.46 \pm 0.09^{f-l}$	$0.38 \pm 0.07^{j-p}$	$0.36 \pm 0.02^{l-p}$	0.38^{C}
	The 12th day	$0.47 \pm 0.08^{e-l}$	$0.40 \pm 0.09^{i-o}$	$0.50 \pm 0.04^{d-k}$	$0.51 \pm 0.06^{d-j}$	$0.46 \pm 0.05^{e-l}$	$0.41 \pm 0.03^{h-n}$	0.46^{B}
	The 15th day	0.70 ± 0.09^{a}	$0.52 \pm 0.12^{c-i}$	$0.59 \pm 0.09^{a-f}$	$0.61 \pm 0.05^{a-d}$	$0.56 \pm 0.10^{b-g}$	$0.55 \pm 0.06^{b-h}$	0.59^{A}
	The 18th day	$0.61 \pm 0.07^{a-d}$	$0.60 \pm 0.09^{a-e}$	0.67 ± 0.04^{ab}	$0.62 \pm 0.07^{a-d}$	$0.54 \pm 0.11^{b-i}$	$0.52 \pm 0.05^{c-i}$	0.59^{A}
	The 21st day	$0.54 \pm 0.15^{b-h}$	$0.59 \pm 0.05^{a-f}$	$0.65 \pm 0.07^{a-c}$	$0.59 \pm 0.05^{a-f}$	$0.50 \pm 0.08^{d-k}$	$0.46 \pm 0.09^{e-l}$	0.56^{A}
	$Mean_{Phe}$	0.48^{B}	$0.45B^{C}$	0.53^{A}	0.53^{A}	$0.46B^{C}$	0.43^{C}	

Values with different letters are significantly different according to the Tukey HSD test at $p < 0.05$. Lowercase letters indicate significant differences in the interaction between culture time and L-phenylalanine concentration, while uppercase letters represent significant differences between treatment



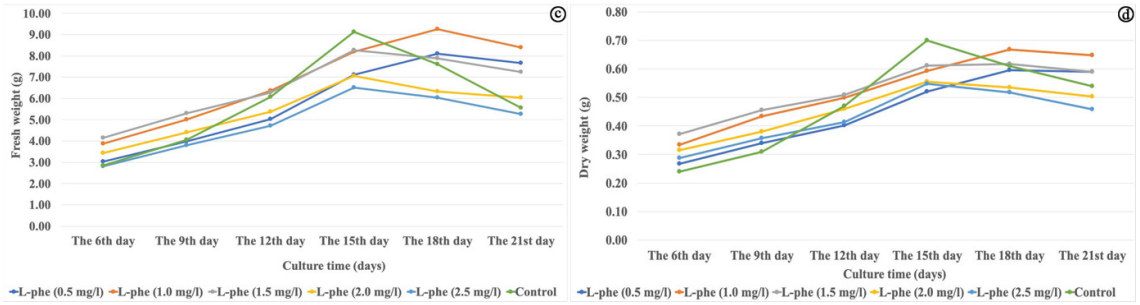


Figure 2. Effects of L-tyr (a, b) and L-phe (c, d) on the biomass of *E. asperula* cells from 6-21 days

Effects of L-tyr and L-phe on TPC and RosA production in E. asperula cells

As described in the materials and methods section, TPC and RosA were quantified at peak biomass and adjacent time points. Therefore, in experiments using 0.5 and 1.0 mg L^{-1} L-tyr, these indices were measured on days 15, 18, and 21, whereas in the control and other L-tyr treatments (1.5 - 2.5 mg L^{-1}), measurements were taken on days 12, 15, and 18.

Supplementation with 0.5 mg L^{-1} L-tyr enhanced biomass production and secondary metabolite accumulation in *E. asperula* cell suspension (Table 1, 3). The TPC and RosA recorded in the experiment using 0.5 mg L^{-1} L-tyr were 75.94 mg GAE g^{-1} DW and 81.98 mg g^{-1} DW, respectively, on the 15th day (Table 3). This result surpasses the control (TPC of 47.74 mg GAE g^{-1} DW and RosA of 43.62 mg g^{-1} DW) for the same culture time. In treatments with higher L-tyr concentrations, both TPC and RosA levels decreased as L-tyr concentration increased. In the experiment with 2.5 mg L^{-1} L-tyr, the TPC and RosA contents were recorded at 51.60 mg GAE g^{-1} DW and 53.33 mg g^{-1} DW, respectively. Although these values were the lowest among the tested treatments, they remained higher than those observed in the control.

Table 3. Effects of L-tyr on TPC and RosA production in *E. asperula* cells

Parameter	Culture time	L-tyrosine (mg L^{-1})					
		Control	0.5	1.0	1.5	2.0	2.5
Total phenolic content (g g^{-1} DW)	The 12th day	24.69 ± 2.88^{b*}	-	-	40.33 ± 2.62^{c}	34.11 ± 2.60^{c}	29.04 ± 2.24^{b}
	The 15th day	47.74 ± 4.17^{a}	75.94 ± 3.62^{a}	58.81 ± 1.46^{b}	60.10 ± 2.64^{a}	60.61 ± 1.26^{a}	51.60 ± 3.32^{a}
	The 18th day	31.93 ± 2.15^{b}	69.69 ± 4.84^{a}	58.55 ± 0.89^{a}	50.95 ± 3.55^{b}	43.95 ± 1.87^{b}	49.08 ± 2.30^{a}
	The 21st day	-	42.11 ± 5.22^{b}	38.51 ± 1.82^{a}	-	-	-
Rosmarinic acid (g g^{-1} DW)	The 12th day	20.37 ± 1.10^{b}	-	-	30.85 ± 3.17^{b}	28.13 ± 1.97^{c}	22.9 ± 2.44^{b}
	The 15th day	43.62 ± 1.58^{a}	81.98 ± 3.89^{a}	55.58 ± 1.03^{a}	57.59 ± 6.18^{a}	56.18 ± 3.95^{a}	53.33 ± 4.42^{a}
	The 18th day	24.60 ± 2.25^{b}	70.74 ± 4.01^{b}	56.28 ± 3.12^{a}	49.87 ± 3.66^{a}	41.21 ± 2.38^{b}	44.80 ± 3.96^{a}
	The 21st day	-	43.07 ± 3.87^{c}	34.99 ± 1.44^{b}	-	-	-

* Means with different letters indicate a statistically significant difference according to the Tukey HSD test at $p < 0.05$ in the same column. "-" data was not recorded

The results presented in Table 2 and Table 4 indicate an inverse correlation between biomass production and the accumulation of these metabolites. Specifically, lower concentrations of L-phe (0.5 and 1.0 mg L^{-1}) promoted higher biomass production but resulted in lower secondary metabolite content. In contrast, higher concentrations of L-phe led to a reduction in biomass but an increase in TPC and RosA content. On the 18th day of culture, which coincided with the peak biomass, the highest TPC achieved in the experiment using 1.0 mg L^{-1} L-phe reached 64.67 mg GAE g^{-1} DW. This value exceeded the result obtained in control on the 15th day of culture, which only reached 47.74 mg GAE g^{-1} DW (Table 4). In the media supplemented with 1.5 and 2.0 mg L^{-1} L-phe, the TPC obtained on the 15th day (when biomass was maximum) exhibited no significant difference, measuring 68.39 mg GAE g^{-1} DW (1.5 mg L^{-1} L-phe) and 68.91 mg GAE g^{-1} DW (2.0

mg L^{-1} L-phe). The distinction between the two treatments only became apparent on the 12th and 18th day post-culture.

On the 18th day of culture, the RosA content in cell suspension grown in medium supplemented with 0.5 mg L^{-1} and 1.0 mg L^{-1} L-phe was not significantly different, measuring 67.66 mg g^{-1} DW and 67.19 mg g^{-1} DW, respectively. These values observed in these two treatments surpassed those of the control obtained at the time of maximum biomass (on day 15 after culture), which measured 43.62 mg g^{-1} DW (Table 4). By day 18, the RosA content in the control had decreased to 24.60 mg g^{-1} DW. Similarly, on the 15th day of culture, the RosA content in experiments using 1.5 mg L^{-1} and 2.0 mg L^{-1} L-phe also showed no significant difference, with measurements of 74.50 mg g^{-1} DW and 75.30 mg g^{-1} DW, respectively. These results indicate that L-phe supplementation at concentrations between 0.5 and 1.0 mg L^{-1} effectively enhanced RosA accumulation in *E. asperula* cell suspension.

Table 4. Effects of L-phe on TPC and RosA production in *E. asperula* cells

Parameter	Culture time	L-phenylalanine (mg L^{-1})					
		Control	0.5	1.0	1.5	2.0	2.5
Total phenolic content (g g^{-1} DW)	The 12th day	24.69 ± 2.88^{b*}	-	-	48.99 ± 3.17^{b}	54.19 ± 2.77^{b}	39.15 ± 5.98^{b}
	The 15th day	47.74 ± 4.17^{a}	57.87 ± 3.46^{a}	56.47 ± 2.93^{ab}	68.39 ± 2.89^{a}	68.92 ± 3.99^{a}	64.25 ± 2.41^{a}
	The 18th day	31.93 ± 2.15^{b}	62.49 ± 5.72^{a}	64.67 ± 7.30^{a}	66.78 ± 3.35^{a}	64.97 ± 3.31^{a}	62.89 ± 3.24^{a}
	The 21st day	-	38.46 ± 3.22^{b}	50.01 ± 4.29^{b}	-	-	-
Rosmarinic acid (g g^{-1} DW)	The 12th day	20.37 ± 1.10^{b}	-	-	43.90 ± 3.23^{b}	47.63 ± 5.28^{b}	32.92 ± 8.98^{b}
	The 15th day	43.62 ± 1.58^{a}	59.39 ± 5.10^{a}	58.70 ± 3.02^{ab}	74.50 ± 5.19^{a}	75.30 ± 1.63^{a}	72.61 ± 4.92^{a}
	The 18th day	24.60 ± 2.25^{b}	67.66 ± 2.16^{a}	67.19 ± 7.51^{a}	76.96 ± 4.52^{a}	79.58 ± 6.32^{a}	68.92 ± 4.22^{a}
	The 21st day	-	34.22 ± 2.86^{b}	53.37 ± 3.02^{b}	-	-	-

* Means with different letters indicate a statistically significant difference according to the Tukey HSD test at $p < 0.05$ in the same column. "-" data was not recorded

The effect of time exposure to precursors on the accumulation of secondary metabolites of E. asperula cell suspension

The types of precursor and the duration of exposure significantly influenced the secondary compound accumulation in *E. asperula* cells. The highest TPC and RosA content were observed in 0.5 mg L^{-1} L-tyr after 24 hours of culture, with TPC of 61.21 mg GAE g^{-1} DW and RosA content of 65.59 mg g^{-1} DW (Figure 3a, b). As the exposure duration to L-tyr was prolonged, the accumulation of TPC and RosA in cells decreased. The TPC and RosA content measured 48 hours post-culture were determined to be 24.85 mg GAE g^{-1} DW and 25.72 mg g^{-1} DW, respectively, and in the experiment with 1.5 mg L^{-1} L-phe, the TPC and RosA content in *E. asperula* cell suspension peaked after 6 hours of culture, reaching 67.04 mg GAE g^{-1} DW for TPC and 71.12 mg g^{-1} DW for RosA content (Figure 3c, d). Notably, this peak occurred sooner than in the treatment with 0.5 mg L^{-1} L-tyr. However, with prolonged exposure to L-phe from 12 to 36 hours, there was a gradual decline in TPC content, with no significant differences observed during these intervals. By 48 hours of culture, the TPC and RosA content decreased to 21.56 mg GAE g^{-1} DW and 20.97 mg g^{-1} DW, respectively, a reduction of over threefold compared to contents measured at 6 hours post-culture.

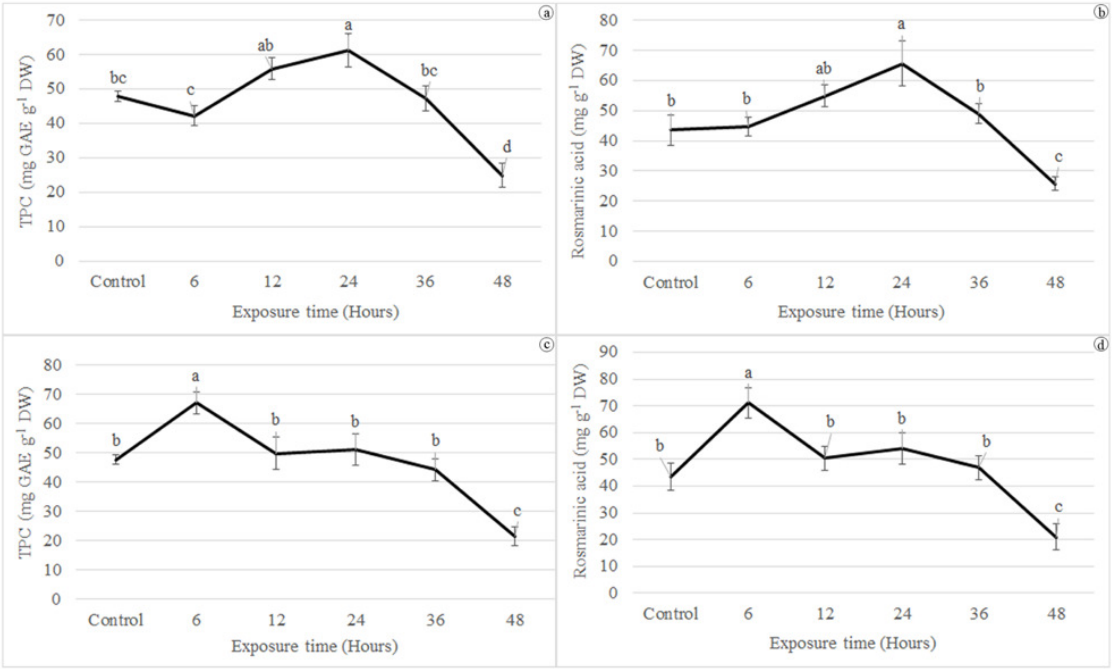


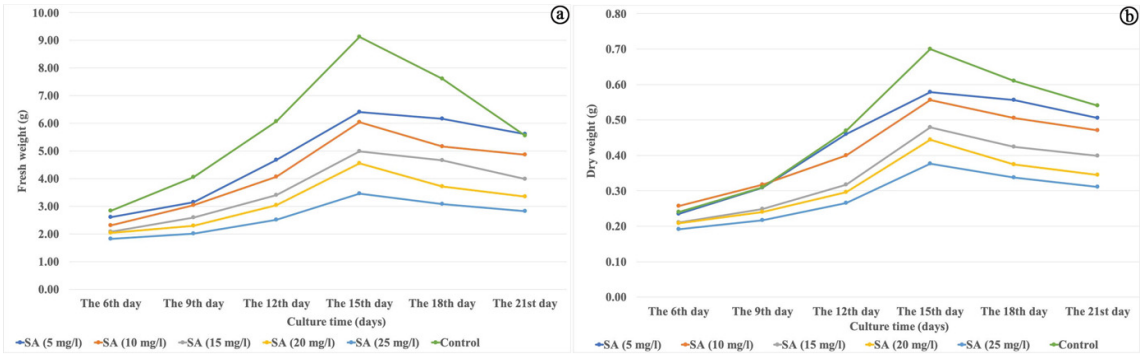
Figure 3. Effect of time exposure to L-tyr (a, b) and L-phe (c, d) on the accumulation of secondary metabolites of *E. asperula* cell suspension

Values present mean ± SD. Bars with different letters differ significantly from each other per Tukey HSD test ($p < 0.05$)

Effects of salicylic acid and yeast extract on the growth of E. asperula cell suspension

A two-way ANOVA model was used to evaluate the effects of elicitors on the FW and DW and culture time of *E. asperula* cell suspensions cultured in control medium (without elicitors) and media supplemented with SA and YE at various concentrations. The results showed statistically significant differences between treatments using SA or YE at different concentrations and culture times ($p < 0.05$).

In the experiments using SA and YE at the concentration tested and the control, the fresh and dry weight measured on the particular days of experiments indicated that SA and YE significantly inhibited the growth of *E. asperula* cell suspensions. This inhibitory effect was particularly pronounced when the elicitors were introduced into the culture medium. The fresh and dry weight in treatments using elicitors were lower than those observed in the control. The exponential phase of cell suspension of *E. asperula* lasted 15 days, followed by the stationary phase until day 18, at which point the cells entered the cellular death phase. The cell suspension growth reached a peak on the 15th day of culture (Figure 4, Table 5).



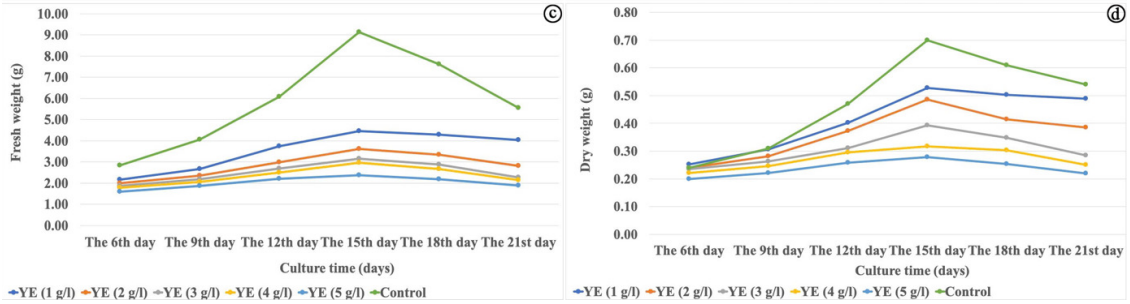


Figure 4. Effects of SA (a, b) and YE (c, d) on the fresh and dry weights of *E. asperula* cell suspensions from 6-21 days

Table 5. Effects of SA on the fresh and dry weight of *E. asperula* cell suspension from 6 to 21 days

Parameter	Culture time (days)	SA (mg L^{-1})						
		Control	5	10	15	20	25	Mean
Fresh weight (g)	The 6th day	2.84 ± 0.41^{l-p*}	2.61 ± 0.38^{m-q}	2.31 ± 0.16^{o-q}	2.09 ± 013^{pq}	2.03 ± 0.12^{pq}	1.82 ± 0.14^{q}	2.29^{D}
	The 9th day	4.05 ± 0.59^{h-j}	3.15 ± 0.45^{k-o}	3.05 ± 0.39^{l-o}	2.60 ± 0.83^{m-q}	2.29 ± 0.12^{o-q}	2.01 ± 0.18^{pq}	2.86C^{D}
	The 12th day	6.07 ± 0.77^{c}	4.67 ± 0.44^{f-h}	4.06 ± 0.88^{h-j}	3.40 ± 0.47^{j-n}	3.04 ± 0.35^{l-o}	2.52 ± 0.26^{n-q}	3.96B^{C}
	The 15th day	9.13 ± 0.50^{a}	6.40 ± 0.177^{c}	6.04 ± 0.36^{cd}	4.99 ± 0.66^{e-g}	4.55 ± 0.27^{g-i}	3.46 ± 0.32^{j-m}	5.76^{A}
	The 18th day	7.61 ± 0.46^{b}	6.16 ± 0.79^{c}	5.17 ± 0.88^{d-g}	4.66 ± 0.22^{f-h}	3.71 ± 0.44^{i-l}	3.08 ± 0.42^{l-o}	5.07^{AB}
	The 21st day	5.56 ± 0.48^{c-f}	5.61 ± 0.83^{c-e}	4.86 ± 0.36^{e-h}	3.99 ± 0.21^{h-k}	3.35 ± 0.16^{j-n}	2.83 ± 0.22^{l-p}	4.37^{B}
	Means	5.87^{A}	4.77^{B}	4.25^{C}	3.62^{D}	3.16^{E}	2.62^{F}	
Dry weight (g)	The 6th day	0.24 ± 0.07^{m-q}	0.23 ± 0.02^{n-q}	0.26 ± 0.02^{l-q}	0.21 ± 0.03^{pq}	0.21 ± 0.01^{pq}	0.19 ± 0.02^{q}	0.22^{F}
	The 9th day	0.32 ± 0.07^{j-n}	0.31 ± 0.04^{j-o}	0.32 ± 0.04^{j-n}	0.25 ± 0.07^{m-q}	0.24 ± 0.01^{n-q}	0.22 ± 0.02^{o-q}	0.27^{E}
	The 12th day	0.47 ± 0.08^{d-h}	0.46 ± 0.04^{e-h}	0.40 ± 0.08^{g-j}	0.32 ± 0.03^{j-n}	0.30 ± 0.03^{k-p}	0.27 ± 0.04^{l-q}	0.37^{D}
	The 15th day	0.70 ± 0.09^{ab}	0.58 ± 0.07^{bc}	0.56 ± 0.03^{b-d}	0.48 ± 0.05^{d-g}	0.44 ± 0.02^{f-h}	0.38 ± 0.05^{h-k}	0.52^{A}
	The 18th day	0.61 ± 0.07^{a}	0.56 ± 0.04^{b-d}	0.51 ± 0.06^{c-f}	0.42 ± 0.02^{f-i}	0.37 ± 0.04^{h-k}	0.34 ± 0.05^{i-m}	0.47^{B}
	The 21st day	0.54 ± 0.15^{b-e}	0.51± 0.05^{c-f}	0.47 ± 0.01^{d-h}	0.40 ± 0.06^{g-j}	0.35 ± 0.02^{i-l}	0.31 ± 0.03^{j-o}	0.43^{C}
	Means	0.48^{A}	0.44^{B}	0.42^{B}	0.35^{C}	0.32^{C}	0.28^{D}	

Values with different letters are significantly different according to the Tukey HSD test at $p < 0.05$. Lowercase letters indicate significant differences in the interaction between culture time and SA concentration, while uppercase letters represent significant differences between treatments

In the experiments using SA, the fresh and dry weight of cell suspensions at all the concentrations tested were lower than those in the control (Table 5). Growth inhibition due to SA supplementation was particularly evident at higher SA concentrations. The highest FW and DW were recorded in the experiment using 5.0 mg L^{-1} SA, with values of 6.40 g and 0.58 g, respectively, on day 15 (Figure 4a, b). In contrast, the FW and DW were lowest in the treatment using 25 mg L^{-1} SA, with values of 3.46 g and 0.38 g, respectively, on the same day.

The addition of YE in the culture medium also inhibited the growth of *E. asperula* cell suspensions. The fresh and dry biomass obtained from YE experiments was significantly lower than that of the control ($p < 0.05$) (Table 6). Results in Table 6 also indicated statistically significant differences among treatments with different culture time ($p < 0.05$). Furthermore, the biomass of the cell suspension decreased as the YE concentration in the culture medium increased. The highest results, with FW of 4.45 g and DW of 0.53 g, were achieved in the 1.0 g L^{-1} YE experiment on day 15 (Table 6, Figure 4c, d).

Table 6. Effects of YE on the fresh and dry weight of *E. asperula* cell suspension from 6 to 21 days

Parameter	Culture time (days)	YE (g L^{-1})						
		Control	1	2	3	4	5	$Means_{CT}$
Fresh weight (g)	The 6th day	$2.84 \pm 0.41^{i\text{-}m*}$	$2.15 \pm 0.35^{m\text{-}q}$	$1.99 \pm 0.12^{o\text{-}q}$	1.86 ± 0.12^{pq}	1.78 ± 0.10^{pq}	1.60 ± 0.11^{q}	2.04^{F}
	The 9th day	$4.05 \pm 0.59^{d\text{-}f}$	$2.67 \pm 0.56^{i\text{-}o}$	$2.34 \pm 0.19^{k\text{-}q}$	$2.19 \pm 0.19^{l\text{-}q}$	$2.06 \pm 0.26^{n\text{-}q}$	1.87 ± 0.29^{pq}	2.53^{E}
	The 12th day	6.07 ± 0.77^{c}	$3.73 \pm 0.47^{d\text{-}g}$	$2.98 \pm 0.30^{g\text{-}k}$	$2.69 \pm 0.34^{i\text{-}o}$	$2.51 \pm 0.22^{j\text{-}p}$	$2.20 \pm 0.35^{l\text{-}q}$	3.36^{C}
	The 15th day	9.13 ± 0.50^{a}	4.45 ± 0.79^{d}	$3.61 \pm 0.28^{e\text{-}h}$	$3.14 \pm 0.34^{g\text{-}j}$	$2.95 \pm 0.14^{h\text{-}l}$	$2.38 \pm 0.30^{j\text{-}p}$	4.28^{A}
	The 18th day	7.61 ± 0.46^{b}	4.29 ± 0.70^{de}	$3.33 \pm 0.57^{f\text{-}i}$	$2.89 \pm 0.34^{h\text{-}m}$	$2.67 \pm 0.43^{i\text{-}o}$	$2.18 \pm 0.20^{m\text{-}q}$	3.83^{B}
	The 21st day	5.56 ± 0.48^{c}	$4.04 \pm 0.72^{d\text{-}f}$	$2.81 \pm 0.37^{i\text{-}n}$	$2.27 \pm 0.35^{k\text{-}q}$	$2.14 \pm 0.58^{m\text{-}q}$	1.89 ± 0.17^{pq}	3.11^{D}
	$Mean_{YE}$	5.88^{A}	3.56^{B}	2.85^{C}	2.51^{D}	2.35^{D}	2.02^{E}	
Dry weight (g)	The 6th day	$0.24 \pm 0.07^{m\text{-}o}$	$0.25 \pm 0.02^{m\text{-}o}$	$0.24 \pm 0.02^{m\text{-}o}$	$0.24 \pm 0.01^{m\text{-}o}$	0.22 ± 0.0^{no}	0.20 ± 0.02^{o}	0.23^{E}
	The 9th day	$0.31 \pm 0.07^{h\text{-}m}$	$0.31 \pm 0.03^{i\text{-}n}$	$0.28 \pm 0.04^{k\text{-}o}$	$0.26 \pm 0.02^{l\text{-}o}$	$0.25 \pm 0.02^{m\text{-}o}$	0.22 ± 0.03^{no}	0.27^{D}
	The 12th day	$0.47 \pm 0.08^{c\text{-}f}$	$0.40 \pm 0.07^{e\text{-}h}$	$0.37 \pm 0.02^{g\text{-}k}$	$0.31 \pm 0.04^{h\text{-}n}$	$0.30 \pm 0.02^{j\text{-}n}$	$0.26 \pm 0.03^{l\text{-}o}$	0.35^{C}
	The 15th day	0.70 ± 0.09^{a}	0.53 ± 0.05^{bc}	$0.49 \pm 0.04^{c\text{-}e}$	$0.39 \pm 0.03^{f\text{-}i}$	$0.32 \pm 0.02^{h\text{-}m}$	$0.28 \pm 0.04^{l\text{-}o}$	0.45^{A}
	The 18th day	0.61 ± 0.07^{ab}	0.50 ± 0.06^{cd}	$0.41 \pm 0.04^{d\text{-}g}$	$0.35 \pm 0.03^{g\text{-}l}$	$0.30 \pm 0.04^{i\text{-}n}$	$0.25 \pm 0.03^{m\text{-}o}$	0.40^{B}
	The 21st day	0.54 ± 0.15^{bc}	$0.49 \pm 0.08^{c\text{-}e}$	$0.39 \pm 0.07^{f\text{-}j}$	$0.28 \pm 0.04^{k\text{-}o}$	$0.25 \pm 0.0^{3m\text{-}o}$	0.22 ± 0.01^{no}	0.36^{C}
	$Mean_{YE}$	0.48^{A}	0.41^{B}	0.36^{C}	0.31^{D}	0.27^{E}	0.24^{F}	

Values with different letters are significantly different according to the Tukey HSD test at $p < 0.05$. Lowercase letters indicate significant differences in the interaction between culture time and YE concentration, while uppercase letters represent significant differences between treatments.

Effects of salicylic acid and yeast extract on TPC and RosA accumulation in E. asperula cell suspension

The study also used a two-way ANOVA model to compare the TPC and RosA content in *E. asperula* cells grown in medium without elicitors and medium with various elicitor concentrations for different durations. The aim was to identify differences in secondary metabolite accumulation based on culture conditions. Biomass was collected on days 12, 15, and 18 post-culture, coinciding with peak biomass and adjacent time points, in order to assess the impact of elicitors on total phenolics and RosA production in cell suspension. The results in Table 7 showed that the supplementation of SA and YE into the media culture of *E. asperula* cell suspension did not affect secondary metabolites accumulation. Additionally, this study observed a positive correlation between fresh weight, dry weight, and the yield of bioactive compounds in relation to elicitor concentration and culture duration. As biomass decreased, the compound yield also declined with

increasing elicitor concentration in the tested treatments. Regarding culture duration, the highest secondary metabolite accumulation was recorded on day 15, coinciding with peak biomass, and decreased by day 18.

Table 7. Effects of SA on TPC and RosA content accumulation in *E. asperula* cell suspension cultures from days 12 to 18

Parameter	Measurement dates	SA (mg L^{-1})						
		Control	5	10	15	20	25	Mean
Total phenolic content (mg g^{-1} DW)	The 12th day	24.69 ± 2.88^{d*}	13.64 ± 1.88^{ef}	4.96 ± 0.77^{h}	7.63 ± 0.22^{gh}	3.00 ± 0.25^{h}	2.65 ± 0.41^{h}	9.43^{B}
	The 15th day	47.74 ± 4.17^{a}	43.39±3.33^{ab}	40.45 ± 2.62^{b}	15.25 ± 0.85^{ef}	7.91 ± 0.31^{gh}	4.11 ± 0.34^{h}	26.48^{A}
	The 18th day	31.93 ± 2.15^{c}	17.41±1.41^{c}	10.86 ± 0.92^{fg}	3.41 ± 0.82^{h}	4.41 ± 0.62^{h}	3.41 ± 0.32^{h}	11.90^{B}
	Means	34.79^{A}	24.81^{B}	18.76^{C}	8.76^{D}	5.11^{E}	3.39^{E}	
Rosmarinic acid (mg g^{-1} DW)	The 12th day	20.37 ± 1.10^{d}	7.53 ± 0.22^{e-g}	4.17 ± 0.66^{gh}	4.65 ± 0.40^{gh}	5.41 ± 0.45^{f-h}	2.41 ± 0.59^{h}	7.42^{B}
	The 15th day	43.62 ± 1.58^{a}	35.20 ± 2.32^{b}	24.31 ± 1.99^{c}	8.52 ± 1.08^{ef}	8.24 ± 1.28^{ef}	6.40 ± 0.33^{fg}	21.05^{A}
	The 18th day	24.60 ± 2.25^{c}	11.02 ± 0.67^{e}	7.68 ± 0.92^{e-g}	4.66 ± 0.60^{gh}	5.35 ± 0.66^{f-h}	6.20 ± 0.29^{fg}	9.92^{B}
	Means	29.53^{A}	17.92^{B}	12.05B^{C}	5.94^{D}	6.33^{D}	5.01^{D}	

Values with different letters are significantly different according to the Tukey HSD test at $p < 0.05$. Lowercase letters indicate significant differences in the interaction between culture time and SAconcentration, while uppercase letters represent significant differences between treatments

The TPC and RosA content in cultures grown in SA-supplemented media were lower than those in the control. A decline in TPC and RosA was associated with increasing SA concentration, with the lowest values of 4.11 mg GAE g^{-1} DW and 6.40 mg g^{-1} DW, respectively, in the 25 mg L^{-1} SA treatment on day 15 (Table 7). This is probably directly connected with the biomass necrosis observed in the experiment (Figure 4). Among SA-treated cultures, the cells grown in 5.0 mg L^{-1} SA exhibited the highest TPC on day 15, reaching 43.39 mg GAE g^{-1} DW. A similar trend was observed for RosA content, with peak levels of 35.20 mg g^{-1} DW recorded in the same treatment on day 15 (Table 7). This experiment revealed a negative correlation between SA concentration and RosA levels, suggesting that high concentrations of salicylic acid may inhibit the accumulation of this important secondary metabolite.

Similar to the SA treatment, YE supplementation did not significantly enhance the accumulation of secondary metabolites in *E. asperula* cells. The highest TPC (54.69 mg GAE g^{-1} DW) and RosA (45.84 mg g^{-1} DW) levels were recorded in the 1.0 g L^{-1} YE treatment. However, TPC and RosA contents in the 1.0 g L^{-1} treatment showed no statistically significant difference compared to both the control and the 2.0 g L^{-1} treatment. Additionally, secondary metabolite levels exhibited a decreasing trend with increasing YE concentration in the culture medium. The lowest values were observed in the 5.0 g L^{-1} YE treatment on day 15, with TPC at 14.15 mg GAE g^{-1} DW and RosA at 7.50 mg g^{-1} DW (Table 8).

The results suggested that YE tended to be more effective than SA in enhancing TPC and RosA accumulation. On day 15 of culture, supplementation with 1.0 mg L^{-1} yielded TPC and RosA contents of 54.69 mg GAE g^{-1} DW and 45.84 mg g^{-1} DW, respectively, which were slightly higher than those obtained with 5.0 mg L^{-1} SA (TPC and RosA were 43.39 mg GAE g^{-1} DW and 35.20 mg g^{-1} DW, respectively), although the difference was relatively minor.

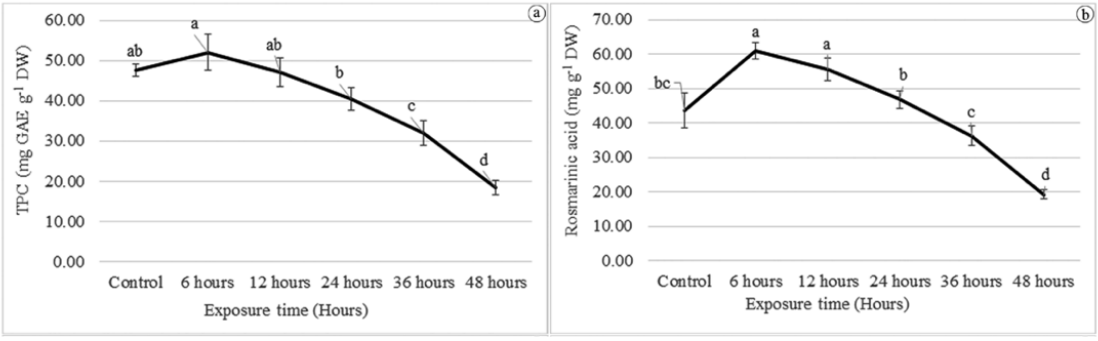
Table 8. Effects of YE on TPC and RosA content accumulation in *E. asperula* cell suspension cultures from days 12 to 18

Parameter	Culture time	YE (g L^{-1})						
		Control	1	2	3	4	5	Mean
Total phenolic content (mg g^{-1} DW)	The 12th day	24.69 ± 2.88^{e-h}	20.90 ± 5.09^{e-i}	17.58 ± 0.81^{e-i}	18.38 ± 4.09^{e-i}	9.67 ± 1.58^{g-i}	7.15 ± 0.05^{i}	16.39^{C}
	The 15th day	47.74 ± 4.17^{ab}	54.69 ± 1.32^{a}	46.57 ± 0.94^{a-c}	30.03 ± 2.78^{c-f}	16.33 ± 2.54^{e-i}	14.15 ± 0.81^{f-i}	34.92^{A}
	The 18th day	31.93 ± 2.15^{b-e}	44.77 ± 6.50^{a-c}	42.23 ± 4.15^{a-d}	26.40 ± 2.53^{d-g}	8.20 ± 1.13^{hi}	7.68 ± 0.14^{i}	26.87^{B}
	Means	34.79^{A}	40.12^{A}	35.46^{A}	24.94^{B}	11.40^{C}	9.66^{C}	
Rosmarinic acid (mg g^{-1} DW)	The 12th day	20.37 ± 1.10^{e-h*}	12.83 ± 2.44f^{i}	10.86 ± 0.98^{f-i}	11.05 ± 1.83^{f-i}	6.25 ± 1.30^{hi}	5.04 ± 0.14^{i}	11.07^{C}
	The 15th day	43.62 ± 1.58^{ab}	45.84 ± 2.85^{a}	38.34 ± 1.45^{a-c}	22.55 ± 3.41^{ef}	11.68 ± 0.68^{f-i}	7.50 ± 0.57^{g-i}	28.26^{A}
	The 18th day	24.60 ± 2.25^{d-f}	38.66 ± 3.65^{a-c}	30.84 ± 1.38^{c-e}	20.65 ± 1.34^{e-g}	4.56 ± 0.51^{i}	4.44 ± 0.35^{i}	20.62^{B}
	Means	29.53^{A}	32.45^{A}	26.68^{A}	18.08^{B}	7.50^{C}	5.66^{C}	

Values with different letters are significantly different according to the Tukey HSD test at $p < 0.05$. Lowercase letters indicate significant differences in the interaction between culture time and YE concentration, while uppercase letters represent significant differences between treatments

The effect of time exposure to salicylic acid and yeast extract on TPC and RosA production in E. asperula cell suspension

The influence of the elicitors (both 5.0 mg L^{-1} SA and 1.0 g L^{-1} YE) on the accumulation of phenolics and RosA in *E. asperula* cells significantly varied depending on the time of exposure after adding these elicitors to the culture medium ($p < 0.05$). The highest levels of TPC and RosA were observed in the cells cultured in the medium containing 5.0 mg L^{-1} SA at 6 hours post-culture, measuring 52.06 mg GAE g^{-1} DW and 60.95 mg g^{-1} DW, respectively, which were not significantly different from the control and 12-hour elicitation treatment (Figure 5a, b). Subsequently, both compounds decreased after 24 and 48 hours of elicitation. The lowest levels were observed 48 hours post-SA treatment, with TPC at 18.50 mg GAE g^{-1} DW and RosA at 19.29 mg g^{-1} DW.



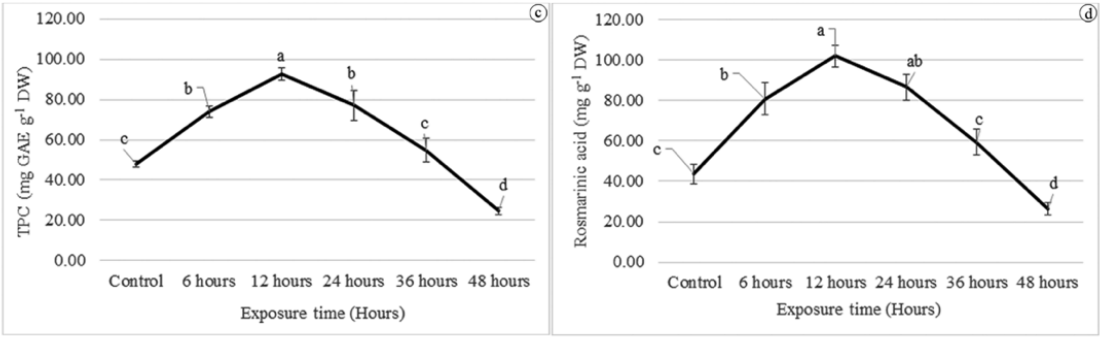
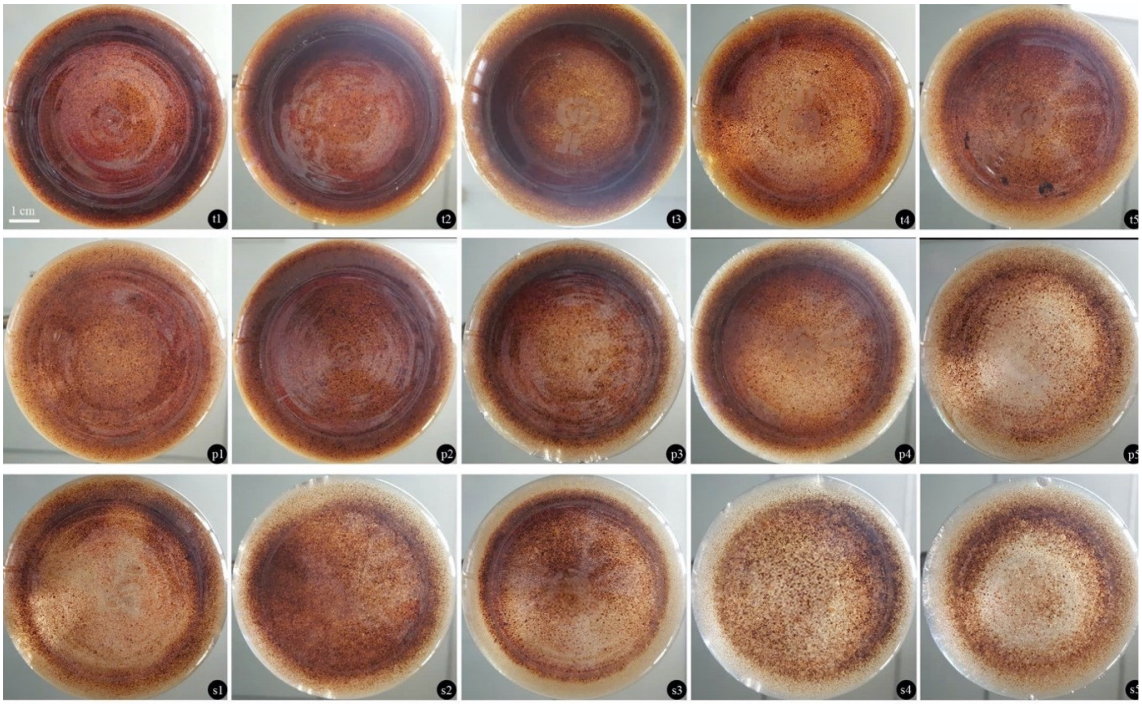


Figure 5. Effect of various exposure times to SA (a, b) and YE (c, d) (including 6, 12, 24, 36, 48 h) on TPC and RosA production in *E. asperula* cell suspension

Values present mean ± SD (n = 3). Bars with different letters differ significantly from each other per Tukey HSD test ($p < 0.05$)

In contrast to 5.0 mg L^{-1} SA experiment, supplementation with 1.0 g L^{-1} YE led to a linear increase in TPC and RosA over the initial 12 hours, peaking at 92.54 mg GAE g^{-1} DW and 101.97 mg g^{-1} DW, respectively, which was a significant difference compared to the control. Following this peak, TPC and RosA content in *E. asperula* cell suspension decreased from 24-48 hours of elicitation, falling to 24.53 mg GAE g^{-1} DW and 26.41 mg g^{-1} DW at 48 hours post-culture (Figure 5c, d). These results indicated that YE supplementation proved more effective in enhancing TPC and RosA accumulation than SA supplementation, with peak values exceeding those obtained with SA elicitation. Notably, YE-elicited cells maintained higher TPC and RosA levels at 48 hours compared to SA-elicited cells.



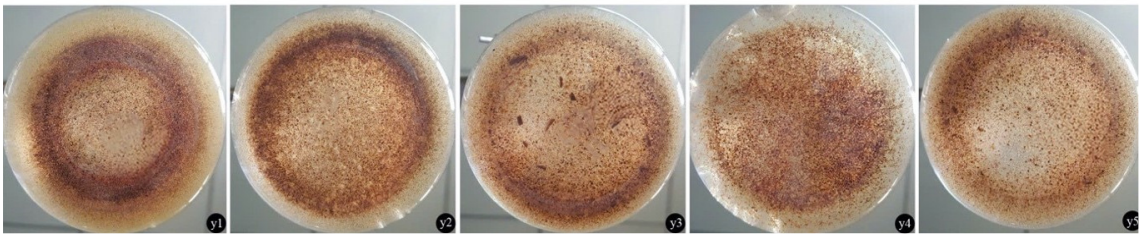


Figure 6. *E. asperula* cell suspension grown in BM medium supplemented with elicitors and precursors BM medium with L-tyr **(t1 - t5)**: Cell suspension were grown in BM medium supplemented with L-tyr at concentrations of **t1** (0.5 mg L^{-1}, day 18), **t2** (1.0 mg L^{-1}, day 18), **t3** (1.5 mg L^{-1}, day 15), **t4** (2.0 mg L^{-1}, day 15), and **t5** (2.5 mg L^{-1}, day 15); BM medium with L-phe **(p1 - p5)**: Cell suspension were grown in BM medium supplemented with L-phe at concentrations of **p1** (0.5 mg L^{-1}, day 18), **p2** (1.0 mg L^{-1}, day 18), **p3** (1.5 mg L^{-1}, day 15), **p4** (2.0 mg L^{-1} day 15), and **p5** (2.5 mg L^{-1}, day 15); SA-supplemented medium **(s1 - s5)**: Cell suspension were grown with SA at concentrations of **s1** (5 mg L^{-1}), **s2** (10 mg L^{-1}), **s3** (15 mg L^{-1}), **s4** (20 mg L^{-1}), and **s5** (25 mg L^{-1}) on the 15th day of culture; YE-supplemented medium **(y1 - y5)**: Cell suspension were grown in YE at concentrations of **y1** (1 g L^{-1}), **y2** (2 g L^{-1}), **y3** (3 g L^{-1}), **y4** (4 g L^{-1}), and **y5** (5 g L^{-1}) on the 15th day of culture

Discussion

Callus morphology plays a critical role in initiating plant cell suspension cultures, and previous studies have highlighted friable callus as the most suitable material. It has been successfully utilized for the establishment of suspension cultures in *Hylocereus costaricensis* (Kong *et al*., 2023) and *Cocos nucifera* L. (Winson *et al*., 2020). Friable calli are particularly advantageous for initiating cell suspension culture due to the ease of cell aggregate disintegration and uniform cell dispersion in liquid medium (Ramulifho, 2019). In this study, calli derived from *E. asperula* young stem explants have a friable morphology, consistent with our previous study (Thao *et al*., 2021). Friable calli of *E. asperula* have likewise been shown to serve as effective initial material for establishing suspension cultures (Thao *et al*., 2021; Tram *et al*., 2022).

E. asperula cells adapted quickly and grew well in the culture medium supplemented with precursors during the first 15 days. Cell growth, indicated by fresh and dry weights, remained unchanged between days 15 and 18, corresponding to the stationary phase. After day 18, most biomass exhibited a considerable decline, marking the onset of the decline phase. However, the growth pattern of cells in media supplemented with low concentrations of L-tyr and L-phe precursors differed from that in high-concentration precursor treatments and the control. This phenomenon can be attributed to the role of L-tyr and L-phe as essential amino acids for plant cell growth and development. These amino acids are indispensable for protein synthesis and various biological functions, directly influencing cellular metabolism and proliferation (Maeda and Dudareva, 2012). Determining the growth process plays a crucial role in biomass cell culture. Understanding the growth phases enables the optimization of subculturing timing, ensuring sustained cell development; the identification of the ideal biomass harvesting stage to maximize compound accumulation; and the strategic supplementation of biomass to enhance culture efficiency. This approach improves overall culture productivity, maximizing biomass yield and secondary metabolite production. Plant cell suspension cultures exhibited varying growth cycles, with some species having short cycles while others extended up to 30 days (Winson *et al*., 2020; Songserm *et al*., 2022).

In this study, the growth pattern of cells in low-concentration L-tyr treatments (from 0.5 to 1.0 mg L^{-1}) exhibited a prolonged exponential phase compared to those in the control experiments and high-concentration L-tyr treatments (from 1.5 to 2.5 mg L^{-1}). The FW and DW in these treatments peaked on day 18 after culture, indicating increased biomass accumulation in these conditions (Figure 6t1, t2). The FW and DW of *E. asperula* cell suspension cultured in the media supplemented with 0.5 mg L^{-1} and 1.0 mg L^{-1} were about 1.26 and 1.11 times higher than the control, respectively. L-tyr feeding enhanced the biomass production and the

accumulation of phenolic compounds in *E. asperula* cells. The 0.5 mg L^{-1} L-tyr treatment was optimal for TPC and RosA accumulation, with TPC levels on day 15 and 18 being 1.59 and 2.18 times higher than the control, while RosA levels were 1.88 and 2.88 times higher, respectively. The results of this study showed that high concentrations of L-tyr in the culture medium inhibited cell growth (Figure 6t4, t5). The reduction in biomass was accompanied by a decline in compound accumulation; however, the compound content remained higher than that of the control. These results were consistent with the study of Hu *et al*. (2014), where the supplementation of 0.75 mM L-tyr increased the PeGs content by 1.60 times compared to the control in the culture of cell suspension of *Cistanche deserticola*. This can be explained by the fact that L-tyr is an essential amino acid that contributes to protein synthesis and serves as a precursor for vital compounds in plant cells, such as vitamin E, phenolic compounds, cyanogenic glycosides, and suberin (Herrmann and Weaver, 1999). Additionally, the role of tyrosine in signal transduction, where it serves as a signaling molecule or an intermediate facilitating interaction between amino acids and phytohormones, thereby regulating endogenous hormone levels (Tegeder and Ward, 2012).

The growth index, such as the fresh and dry weight, revealed a more extended exponential growth phase for *E. asperula* cells cultured in a medium with low L-phe concentrations than those grown in a medium with higher L-phe concentrations (Figure 6p1-p5). Similar to the L-tyr treatment, compound accumulation peaked on day 18, coinciding with the highest biomass in the 0.5 and 1.0 mg L^{-1} L-phe treatments, while in the higher L-phe concentrations (1.5 - 2.5 mg L^{-1}), the peak occurred earlier, on day 15. Our results showed that elevated L-phe concentration in the culture medium increased the TPC and RosA content accumulated in *E. asperula* cells. In media supplemented with 1.5 and 2.0 mg L^{-1} L-phe, the RosA content obtained on day 15 was 1.71 - 1.73 times higher than the control.

The experimental results indicate no direct correlation between increased cell biomass and the accumulation of secondary metabolites. Specifically, in treatments supplemented with 0.5 and 1.0 mg L^{-1} L-phe, although cell biomass reached its highest levels, the accumulation of secondary metabolites remained low. In contrast, at higher L-phe concentrations, cell biomass decreased, whereas the accumulation of secondary metabolites increased. Adams *et al*. (2019) studied the regulation of phenylalanine metabolism pathways through the presence of shikimate (a precursor of L-phe biosynthesis) and showed that when shikimate is present in cells at low concentrations (corresponding to low pre-existing phenylalanine concentrations), phenylalanine is directed towards primary metabolism processes (such as protein synthesis). When the shikimate concentration increases and the threshold constants of the primary metabolic pathway become saturated, phenylalanine is directed toward the secondary metabolic pathway. This hypothesis also explains the results of this study: L-phe is supplemented at a low concentration in the medium, and this precursor is preferentially used for the primary metabolic pathway, promoting increased cell biomass, extending the cell growth cycle, and reducing the accumulation of secondary metabolites within *E. asperula* cells. Consistent with our findings, previous studies have also demonstrated the positive effect of precursor supplementation on secondary metabolite accumulation. For example, Sahraroo *et al*. (2018) reported that L-phe supplementation reduced explant growth but enhanced RosA accumulation in *Satureja khuzistanica* hairy roots, with a maximum content of 227.76 mg g^{-1} DW at 3.0 mM L-phe. Similarly, Shinde *et al*. (2009) noted that precursors are essential molecules in metabolite biosynthesis, and their supplementation effectively enhances the production of target metabolites. These observations reinforce the conclusion that L-phe supplementation can significantly promote RosA production in plants.

The study found that adding L-tyr and L-phe to the culture medium significantly affected the growth and accumulation of secondary compounds in the *E. asperula* cells. The optimal concentrations for maximizing secondary compound production were identified as 0.5 mg L^{-1} for L-tyr and 1.5 mg L^{-1} for L-phe. As with previous research, it's essential to assess the duration of cell exposure to these precursors and effectively utilize them to determine the best approach for increasing the secondary compound content accumulated in *E. asperula* cells. TPC and RosA contents varied depending on the exposure time to the precursors, and there were

significant differences between the two experiments tested (0.5 mg L^{-1} L-tyr and 1.5 mg L^{-1} L-phe). While the TPC and RosA content of cells cultured in the medium supplemented with 0.5 mg L^{-1} L-tyr peaked after 24 hours, in the experiment using 1.5 mg L^{-1} L-phe, TPC and RosA content in cells reached their highest after only 6 hours of culture. However, extending the exposure time to L-tyr or L-phe beyond 48 hours significantly reduced TPC and RosA content in *E. asperula* cells. After 48 hours of culture, TPC and RosA content decreased by 2.46 and 2.55 times compared to the 24-hour measurement. Similarly, increasing L-phe exposure from 12 to 36 hours led to a significant decline in TPC and RosA. By 48 hours, TPC and RosA decreased more than threefold compared to the 6-hour measurement. This result contradicts the results of Dewanjee *et al*. (2014), who reported that L-phe (100 mg L^{-1}) and L-tyr (400 mg L^{-1}) enhanced the accumulation of RosA in *Solenostemon scutellarioides* cells, 1.5 and 2.1 times higher than control, respectively, after 48 hours. Feduraev *et al*. (2020) reported that 800 µM L-tyr and 400 µM L-phe enhanced phenolic accumulation in *Triticum aestivum* L cells after 4 hours of culture. The concentration of L-phe, L-tyr and exposure time remained critical factors, which vary with species and metabolites (Dewanjee *et al*., 2014).

As mentioned earlier, with the goal of effectively utilizing precursors or elicitors to enhance the production of secondary metabolites in *E. asperula* cells, this study investigated whether the difference between long-term exposure to precursors/elicitors is more beneficial than short-term exposure in TPC and RosA content accumulation in cells. Interestingly, there was no significant difference in the amounts of TPC and RosA accumulated between the two methods. For instance, cells continuously exposed to L-tyr (0.5 mg L^{-1}) for 15 days produced less phenolic and RosA than those given a single 24-hour dose of the same concentration. This pattern was also observed for both the tested L-phe and elicitor experiments. These findings suggest that short-term exposure to precursors is as adequate as constantly adding them to boost the production of desired secondary metabolites in *E. asperula* cell cultures. This approach could lead to more efficient use of elicitors/precursors and optimized culture medium in producing phenolics and RosA by the *E. asperula* cell suspension culture.

The latest trend involves stimulating *in vitro* secondary metabolite biosynthesis through an elicitor. Elicitor-induced phenolic and RosA biosynthesis is influenced by elicitor type, concentration, and duration of exposure (Açıkgöz, 2020). Our study demonstrated distinct effects of the abiotic elicitor (SA) and the biotic elicitor (YE) on biomass production and the accumulation of secondary metabolites in *E. asperula* cells. Both SA and YE negatively influenced cell growth, as evidenced by the significantly lower fresh and dry weights in all elicitor-treated explants compared to the control. Following the determination of *E. asperula* cell growth dynamics in elicitor-supplemented media, the accumulation of TPC and RosA was measured at the peak biomass time point and two adjacent time points. The peak TPC and RosA content generally coincided with the late exponential phase (day 15) across all treatments, including the control. Throughout the growth cycle, fluctuations in secondary metabolite content were influenced by biomass dynamics, with content increasing as biomass expanded and decreasing as it declined. Notably, SA and YE exhibited significantly different effects on secondary metabolite accumulation in *E. asperula* cell suspension.

Regarding SA's role in plant growth remains controversial, its growth-inhibitory effects and capacity to induce secondary metabolite biosynthesis are evident (Vicente and Plasencia, 2011). In the SA experiment, the growth index, such as fresh and dry weight, was lower than in the control. The growth of *E. asperula* cell suspension was significantly decreased with an increase in SA concentration (Figure 6s1-s5). This result was consistent with the findings reported by Quang *et al*. (2022). The growth of Giao co lam (*Gynostemma pentaphyllum* (Thunb.) Makino) cells also decreased with an increase in SA concentration (Quang *et al*., 2022). In this regard, our results are also consistent with observations from other cell cultures, including *Lonicera japonica* (Du *et al*., 2020) and *Crocus sativus* (Moradi *et al*., 2020), where the application of SA as an elicitor resulted in the inhibition of cell growth.

In contrast to the precursor experiments or the SA studies mentioned above, our observations showed that the SA concentration employed not only decreased biomass production but also inhibited the accumulation of secondary compounds. The TPC and RosA content in cells cultured in a medium supplemented with SA at 5 - 25 mg L^{-1} concentrations were lower than those in the control, except for the 5 mg L^{-1} SA treatment, in which the secondary metabolites content did not significantly differ from the control. SA concentration was a crucial factor influencing bioactive compound production in plant species; the tested SA concentration must be appropriate for each plant species and tissue explants (Khalil *et al*., 2022). Low SA concentrations may be insufficient to activate physiological processes and regulate gene expression for secondary metabolite biosynthesis, whereas high SA concentrations often induce detrimental effects (Ahmed *et al*., 2022). The results in this experiment contrast with the study by Dong *et al*. (2010), where SA was found to enhance the accumulation of phenolic compounds by activating PAL gene expression in *Salvia miltiorrhiza* cells. *Origanum vulgare* cells elicited with 200 µM SA (about 27.624 mg L^{-1} SA) exhibited the highest TPC (3.80-fold increase) and RosA (4.17-fold increase) compared with the control (Li *et al*., 2021). Al-Khayri *et al*. (2020) identified the MS medium supplemented with 50 mg L^{-1} SA as optimal for increasing TPC in *Phoenix dactylifera* cells. The optimal SA concentration for maximizing the accumulation of most compounds in *Ajuga integrifolia* shoots was 150 µM SA (Abbasi *et al*., 2020). These findings highlight the differential responses of various plant species and organs to varying SA concentrations.

Compared with SA, YE supplementation significantly enhanced TPC and RosA accumulation in *E. asperula* cell suspensions, with the highest TPC observed at 1.0 g L^{-1} YE, consistent with previous findings in *Zataria multiflora* (Bavi *et al*., 2022). TPC in *Zataria multiflora* cells treated with 1.2 g L^{-1} of YE was 36% higher than the control (Bavi *et al*., 2022). Beyond phenolics, YE elevated RosA content across diverse plant species and culture systems, including cell suspensions of *Agastache rugosa* (Park *et al*., 2016), *Origanum vulgare* (Li *et al*., 2021), and *Lavandula angustifolia* (Yazdi *et al*., 2023). Regarding its mode of action, YE did not directly influence secondary metabolite biosynthesis; it stimulated the production of endogenous jasmonic acid and methyl jasmonate, thereby indirectly enhancing secondary metabolite accumulation (Sánchez-Sampedro *et al*., 2005).

On the other hand, Bavi *et al*. (2022) reported elevated H_2O_2 and NO levels in YE-treated *Z. multiflora* cells. Oxidative stress triggers the phenylpropanoid pathway, upregulating phenylalanine ammonia-lyase (PAL) and tyrosine ammonia-lyase (TAL) gene expression, consequently enhancing phenolic compound production. However, in YE-supplemented media, elevated H_2O_2 and NO levels induced oxidative stress, leading to cell membrane damage and a consequent reduction in biomass in *Zataria multiflora* cells (Bavi *et al*., 2022). Building on these findings, our study provides further evidence of the negative impact of YE supplementation on plant cell growth. Specifically, the results showed that YE supplementation significantly decreased both the fresh and dry weight of *E. asperula* cells (Figure 6y1-y5). This result is consistent with previous reports of growth inhibition in various plant species, including *Salvia miltiorrhiza* (Zhao *et al*., 2010), *Z. multiflora* (Bavi *et al*., 2022)... However, species-specific responses to YE have been documented (Vasil and Hildebrandt, 1966). Our results contrast with those of Tuan *et al*. (2017), who reported increased biomass in *Panax vietnamensis* with YE treatment. Similarly, Deepthi and Satheeshkumar (2016) found a significant increase in the biomass of *Ophiorrhiza mungos* grown in cell suspension culture with YE supplementation.

These results highlight the differential effects of SA and YE on the growth and secondary metabolite accumulation of *E. asperula* cells and provide valuable insights for optimizing the culture conditions for specific applications. However, the early addition of elicitors and prolonged exposure had a detrimental effect on cell growth, even at minimal concentrations. According to Thakur and Sohal (2013), elicitor-initiated defence signaling cascades result in the overproduction of reactive oxygen species (ROS) and the ensuing hypersensitivity response (HR). This culminated in programmed cell death, manifesting as diminished viability, reduced biomass, and accumulation of secondary metabolites within the cell. Therefore, to overcome

this situation, it is necessary to shorten the exposure time with elicitors to optimize the yield of secondary compounds from the cells. Our results indicated that short-term elicitor treatments were more efficient in inducing the accumulation of TPC and RosA in *E. asperula* cells. Furthermore, in nature, the accumulation of secondary metabolites can be stimulated by the presence of stress factors (both biotic and abiotic stress), which helps plants enhance their resilience against stress. However, these environmental factors are often unpredictable in terms of intensity and duration, leading to fluctuating secondary metabolite accumulation. Similarly, *in vitro* secondary metabolite production is influenced by type, concentration, and time exposure to stress factors, especially elicitors. This study also showed that TPC and RosA content changed depending on the elicitor type and exposure time to the elicitor. YE added to the *E. asperula* cell suspension culture medium had a better effect on inducing RosA and phenolic accumulation in cells than SA. At 6 hours of culture, the TPC and RosA contents in cells grown in medium supplemented with 1.0 g L^{-1} YE had not yet reached their maximum, but they were still higher than those observed in cells cultured with 5.0 mg L^{-1} SA at the same time point.

In the experiments involving elicitors (both SA and YE), which initially enhanced the accumulation of secondary metabolites in *E. asperula* cells, the TPC and RosA reached their highest levels at 6 hours for 5 mg L^{-1} SA and 12 hours for 1.0 g L^{-1} YE, respectively. However, prolonged exposure led to a decline in these compounds. The rapidly increasing secondary metabolite content inhibited the growth of *E. asperula* cells because these compounds are not essential for cell growth. When secondary metabolite content is too high, it can be toxic to the cells, causing functional disturbances and a subsequent decline in secondary metabolite content. A comparison of these two experiments indicates that eliciting *E. asperula* cell suspension cultures with YE on day 15 and harvesting after 12 hours is an effective strategy for maximizing secondary metabolite production. This result is also consistent with the study by Sahu *et al*. (2013), who demonstrated that YE enhanced RosA accumulation in *S. scutellarioides* cells after 12 hours of treatment. Yeast extract components, including nucleotides, β-glucans, and mannan oligosaccharides, may act as elicitors, bind to plant cell receptors, and trigger signaling pathways that lead to secondary metabolite production (Portu *et al*., 2016). In addition, YE are considered safe for inclusion in food and pharmaceutical products with minimal environmental concerns (Portu *et al*., 2016).

Conclusion

The addition of specific precursors and elicitors, such as L-tyr, L-phe, SA, and YE, activated the secondary metabolites biosynthesis pathway in *E. asperula* cells. In the *E. asperula* cell suspension cultures, precursor feeding, such as L-tyr and L-phe, was more efficient than elicitor supplementation in improving the biomass production as well as TPC and RosA accumulation. L-tyr (0.5 mg L^{-1}) and L-phe (1.5 mg L^{-1}) promoted the highest TPC and RosA content accumulation. These findings suggest that TPC and RosA biosynthesis in *E. asperula* cells depends on L-tyr and L-phe as precursors, proceeding via the phenylpropanoid and tyrosine pathways. Adding 0.5 mg L^{-1} L-tyr proved to be the optimal concentration for simultaneously enhancing cell growth and TPC and RosA production. *E. asperula* cells biomass obtained through cell suspension culture is a rich source for producing bioactive secondary compounds, such as TPC and RosA. They may be safe for producing pharmaceuticals, cosmetics, and functional foods.

Authors' Contributions

Conceptualization: TTT, and HTT; Data curation: TT.; Formal analysis: HTT, and GDD; Funding acquisition: TTT; Investigation: TTVN, HTTN, TKTD and THTN; Methodology: TTT, and HTT;

Project administration: TTT and GDD; Supervision: HTT and TTT; Visualization: TTVN and TTT; Writing original draft: TTT and HTT; and Writing - review & editing: TTT, HTT All authors read and approved the final manuscript.

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Conflict of Interests

The authors declare that there are no conflicts of interest related to this article.

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