Trop J Nat Prod Res, August 2021; 5(8):1403-1408
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

LC-MS Based Secondary Metabolites Profile of *Elaeocarpus grandiflorus* J.E. Smith. Cell Suspension Culture Using Picloram and 2,4-Dichlorophenoxyacetic Acid

Noor A. Habibah*, Yustinus U. Anggraito, Nugrahaningsih Nugrahaningsih, Safitri Safitri, Fajar Musafa, Nur Wijayati

Biology Department, Faculty of Mathematics and Sciences, Universitas Negeri Semarang, Indonesia

ARTICLE INFO

Article history:
Received 08 May 2021
Revised 16 June 2021
Accepted 26 August 2021
Published online 02 September 2021

ABSTRACT

Elaeocarpus grandiflorus contains prominent bioactive compounds. The bioactive metabolites can be increased using the cell suspension culture technique by adding synthetic auxin, including picloram and 2,4-dichlorophenoxyacetic acid (2,4-D). Therefore, this study aimed to analyze the effect of picloram and 2,4-D on the secondary metabolite profile of *E. grandiflorus* cell suspension culture. Petioles of young leaves from *E. grandiflorus* were used as explants for callus induction, and then the callus was used for cell suspension culture. The cell culture was maintained on a woody plant medium (WPM) for 30 days supplemented with picloram (3.5 mg/L; 5.0 mg/L; and 7.5 mg/L), or 2,4-D (1.5 mg/L; 2.5 mg/L; and 3.5 mg/L). The 2.5 mg/L 2,4-D treatment with the highest dry weight was harvested every five days until the 30th day. Secondary metabolites in all treatments showed no significant difference (P = 0.949, $F_{3.6}$ = 0.228), and the highest content of secondary metabolites was kaempferols which was up to 24.29 ± 0.77%, while the total average flavonoid content was up to 55.69 ± 0.96%. In addition, the secondary metabolites did not change significantly for 30 days (P = 0.974, $F_{3.6}$ = 0.279). Most plant energy and hormones were used for cell division and growth instead of secondary metabolite biosynthesis during this period. This study showed that picloram and 2.4-D induction have no significantly different effect on the secondary metabolite profile in the *E. grandiflorus* cell suspension culture.

Keywords: Auxin, Bioactive compound, Flavonoid, LC-MS, Rejasa.

Introduction

Elaeocarpus grandiflorus J. E. Smith is a member of the Elaeocarpaceae family, Magnoliopsida, known as *rejasa* or *anyang-anyang* (vernacular name) in Indonesia. This plant is a flora of regional identity with a declining population.[1] Generally, species from Elaeocarpaceae are reported to contain active compounds such as alkaloids, flavonoids, glycosides, tannins, terpenoids, and phenolic acids.[2,3] *Elaeocarpus's* bark has potential as an antidiabetic drug.[4] Furthermore, the leaves can be used in herbal medicine as tonics, diuretics, and fever relievers.[5] It is a potential HIV-1 protease inhibitory drug candidate, that prevents the virus from replicating.[6]

The conservation status continuously shows a declining population of *E. grandiflorus*, and its abundance of potentially bioactive compounds makes studying this species essential. Furthermore, cell suspension culture is an effective strategy for increasing secondary metabolic production and preventing overexploitation of protected plant species.[7–9] The methods are safe, economical, accessible, and straightforward compared to taking secondary metabolite directly from the plant tissue. Secondary metabolites contents obtained from the cell suspension culture process can also be optimized using synthetic auxin induction, such as by adding 4-amino 3,5,6-tricloropicolinic acid (picloram) or 2,4-dichlorophenoxyacetic acid (2,4-D).[10] Picloram and 2,4-D are powerful auxin-herbicides used as a plant growth regulator (PGR) to increase flavonoid content in the cell culture.[11,12]

*Corresponding author. E mail: nooraini@mail.unnes.ac.id
Tel: +6281548844310

Citation: Noor AH, Yustinus U.A, Nugrahaningsih WH, Safitri S, Fajar M, Nur W. LC-MS Based Secondary Metabolites Profile of *Elaeocarpus grandiflorus* J.E. Smith. Cell Suspension Culture Using Picloram and 2,4-Dichlorophenoxyacetic Acid. Trop J Nat Prod Res. 2021; 5(8):1403-1408. doi.org/10.26538/tjnpr/v5i8.13

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

Meanwhile, the treatment with 7.5 mg/L picloram or 2.5 mg/L 2,4-D in *E. grandiflorus* culture media grows calli optimally and increases the total phenol concentration as a leading bioactive compound group.[13]

High bioactive productivity, represented by secondary metabolite profile including content composition and concentration. Optimization of the picloram and 2,4-D concentration in the cell cultures still needs to be conducted. It is necessary to determine the optimal doses of synthetic-auxin to increase secondary metabolite productivity in *E. grandiflorus* cell suspension cultures. Therefore, this study aimed to analyze the effect of picloram and 2,4-D on the secondary metabolite profile of *E. grandiflorus* cell suspension cultures.

Materials and Methods

This research was an experimental study using *E. grandiflorus* cell suspension culture. A 2-year-old *E. grandiflorus* plant was collected from Bali Botanical Garden, Indonesian Institute of Sciences, Bali, Indonesia. A voucher specimen (No: HS–2020–07–016) was deposited in the Plant Culture Laboratory, Biology Department, Universitas Negeri Semarang, Central Java, Indonesia. Sample of petioles leaves were collected in June 2020 from this single plant. A total of six experimental treatments consisting of *E. grandiflorus* cells were conducted using picloram and 2,4-D at different concentrations following previous studies.[12,14]

Culture media for cell suspension culture

This research was conducted in the Plant Culture Laboratory, Biology Department, Universitas Negeri Semarang. Cell suspension media was made using McCown's woody plant basal salt mixture (WPM) Cat. No: M6774–10L (Sigma–Aldrich: Jakarta, Indonesia) supplemented with picloram or 2,4-D. The medium solution was divided into six Erlenmeyer flasks, and 3.5 mg/L, 5 mg/L, and 7.5 mg/L picloram or 1.5 mg/L, 2.5 mg/L, and 3.5 mg/L 2,4-D was added. Three percent sucrose was added to each medium solution, and the pH was adjusted to 5.8. Then, a total of 20 mL of each media solution was poured into

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

a 100 mL Erlenmeyer flask and it was tightly closed. It was sterilized in an autoclave at a temperature of 121°C and pressure between 1.1–1.5 kg/cm^2 for 20 minutes.

Callus and cell culture induction

The explants were collected from young petiole leaves 3–5 from the shoots. The petioles were managed aseptically, then sterilized using a fungicide, bactericide, and 5.25% NaClO solution following the procedure reported by Habibah *et al*.[12,14] The petiole pieces were placed on a WPM agar medium and incubated at 26°C for five months in dark conditions. The produced calluses were used as a material for the induction of cell culture. The formation of cell suspension culture was performed by transferring calli into a 100 mL Erlenmeyer tube containing 20 mL of WPM medium with various PGR concentrations. The culture was shaken at 100 rpm, then incubated and maintained for 30 days in the dark condition. The highest mass of cultured cells was achieved in the 2.5 mg/L 2,4-D treatment. It was harvested regularly every five days up to the 30th day to evaluate the secondary metabolism profile. After harvesting, the cells were filtered and dried in an oven for 48 h at 60 °C. The dried cells were weighed and extracted for LC–MS analysis.

Cell and secondary metabolites extraction

The secondary metabolites were extracted following a procedure modified from Hao *et al*.[15] The cell cultures were dried and ground into powder using a mortar and pestle. The fine cell powder was used for secondary metabolite extraction using 5 mL of methanol containing 1% HCl (v/v) and 5 mL of 2 N HCl was added. Then, the solution was incubated for 1 h at 90ºC in the thermal incubator. The extract solution was dried and resuspended in methanol.

LC–MS Analysis

The supernatant from the finished extraction stage was put into a Sep-Pak C18 Cartridge (1 cc, 100 mg) that had been conditioned with 1 mL 80:20 of acetonitrile-water (v/v). A total of 0.5 mL of the solution that comes out was then collected, and 1 mL of protein precipitation sample was added into the Sep-Pak C18 column. The test sample was then added 0.25 mL of 200 mM ammonium formate (NH_4HCO_2) in a 50:50 of acetonitrile-methanol solution (v/v) into the Sep-Pak column. A total of 0.5 mL of the solution that came out was collected and added with 0.2 mL of 25:75 acetonitrile-buffer solution. (25 mM of ammonium formate, pH 4.5). The solution was filtered with a membrane filter Whatman® cellulose acetate 0.45 µm, then degassed and injected into the LCMS machine.

The LC–MS machine used in this study was the LC–MS apparatus model of the Shimadzu LCMS - 8040 LC/MS (Shimadzu: Kyoto, Japan), using Shimadzu Shim Pack FC-ODS (2 mm x 150 mm, 3 µm) column in 35 °C with an injection volume up to 1 µL. The LC–MS machine uses a capillary voltage of 3.0 kV, an isocratic mobile phase mode, and a 0.5 mL/min flow rate. The collision energy used was 5.0 V, 60 mL/hour for desolvation gas flow at 350 °C. The scanning process runs at a speed of 0.6 sec/scan (Mz: 10 –1000), a source temperature of 100 °C, and a run time of 80 minutes.

Statistical analysis

The secondary metabolite concentration was stated as a percentage value and analyzed using one-way ANOVA followed by the least-significant difference (LSD) test with a confidence interval of 95%. All statistical analysis were performed using SPSS. v23.

Results and Discussion

The effect of picloram or 2,4-D induction in cell suspension cultures did not significantly differ in the percentage of secondary metabolite productivity but otherwise increased the dry weight. In addition, there was also no significant increase in the secondary metabolite concentration after 30 days. This result implies that during 30 days of culture, phytohormone induction may only affect the mass growth in *E. grandiflorus* cell suspension culture. More time may be needed more time for suspension culture to produce secondary metabolites.

The growth rate of the *E. grandiflorus* cell suspension culture increased on the 15th day and decreased on the day after (Figure 1), but the maximal dry weight was reached on the 30th day. Meanwhile, according to Habibah *et al*.,[16] the highest biomass can be obtained after 30 days of treatment in *Stelechocarpus burahol* cell cultures. Then, the lag (growth) phase of *S. burahol* cell suspension culture was observed for the first six days, followed by the log phase from six to 30 days. Additionally, the cells reached the stationary phase after 30-36 days of treatment, but the highest biomass was obtained on the 30th day.[16]

A high mass of fresh weight but low biomass synthesis of *E. grandifloras* cell suspension may correlate with cell division and cytoplasm content in the early growth step. In contrast, it started producing more biomass and organic materials for development after 15 days of culture. This was shown by the decrease of fresh weight on the 20th to 30th day but increased dry weight.

Several studies have shown that the production of secondary metabolites increases simultaneously alongside the dry-weight after synthetic auxin application. Picloram and 2,4-D effectively increase secondary metabolite production, especially flavonoids and phenolic acids.[15,16] Most of the phytohormone addition to *Digitalis davisiana* cell culture increases digoxin and lanatoside C synthesis.[17] However, in this study, the application of picloram and 2,4-D had no different effect on secondary metabolite productivity (P = 0.000), as presented in Table 1.

E. grandiflorus cell suspension culture induced with 3.5 mg/L 2,4-D gave the highest level of secondary metabolites, although it was not significantly different from picloram induction or 2,4-D at lower doses (P = 0.949, $F_{3.6}$ = 0.228). Separately, secondary metabolite compositions also showed insignificant results in all treatments (P> 0.050). The concentrations of secondary metabolites, including flavonoids, showed no significant difference among the treatments (P = 0.974, $F_{3.6}$ = 0.279). Nevertheless, induction using 3.5 mg/L 2,4-D resulted in the highest percentage of secondary metabolites concentration and the most dominant compound. The kaempferol component had the most abundant secondary metabolite production as a flavonoid group, especially in the low-dose picloram treatment (Table 2).

Low concentration of picloram or 2,4-D may increase secondary metabolite production, including phenolic acids and tannins. A similar effect was also observed for alkaloids and terpenoid group compounds. Supporting the result, applying low picloram concentrations can stimulate DNA, RNA, and protein synthesis for the cell to conduct mitosis and growth. Meanwhile, high concentrations of picloram can act as cell division inhibitor.[15]

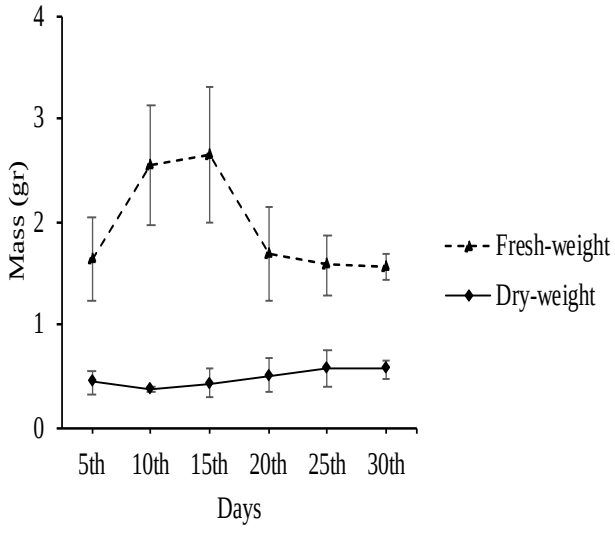

Figure 1: The *E. grandiflorus* cell suspension mass during the culture step for 30 days.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Secondary metabolite product per component group in the *E. grandiflorus* cell suspension culture on WPM medium with the addition of picloram and 2,4-D.

Compound	**Concentration (%)**						
Groups	NTC	PC.1	PC.2	PC.3	2,4-D_1.5	2,4-D_2.5	2,4-D_3.5
Alkaloids	12	7.13 ± 0.13	6.72 ± 0.13	7.11 ± 0.15	6.83 ± 0.13	6.67 ± 0.12	7.25 ± 0.11
Dicarboxylic acid	5	3.65 ± 0.19	5.27 ± 0.13	5.50 ± 0.14	5.36 ± 0.15	5.32 ± 0.19	4.31 ± 0.08
Flavonoid	32	56.92 ± 0.20	54.81 ± 0.19	56.19 ± 0.19	54.33 ± 0.20	55.76 ± 0.19	56.15 ± 0.19
Phenolic acid	7	12.15 ± 0.64	12.03 ± 0.63	12.00 ± 0.62	12,49 ± 0.00	11.90 ± 0.00	11.89 ± 0.00
Phytosterol	1	0.27 ± 0.00	0.30 ± 0.00	0.27 ± 0.00	0.28 ± 0.00	0.27 ± 0.00	0.27 ± 0.00
Tannin	1	2.75 ± 0.00	2.70 ± 0.00	2.72 ± 0.00	2.83 ± 0.15	2.70 ± 0.14	2.71 ± 0.15
Terpenoid	18	6.20 ± 0.14	6.48 ± 0.15	5.93 ± 0.14	6.34 ± 0.14	5.88 ± 0.14	6.09 ± 0.15
Vitamin	10	3.28 ± 0.18	3.77 ± 0.18	3.48 ± 0.17	3.63 ± 0.18	4.07 ± 0.25	3.36 ± 0.18
Other compounds	10	7.65 ± 0.50	7.92 ± 0.48	7.80 ± 0.48	7.89 ± 0.51	7.43 ± 0.42	7.96 ± 0.48

Note: NTC = number of a total components. PC1-3 represents the treatment with 2.5 mg/L, 5 mg/L, and 7.5 mg/L of picloram, respectively. Then 2,4D 1-3 represents the treatment with 1.5 mg/L, 2.5 mg/L and 3.5 mg/L of 2,4-D, respectively.

On the other hand, the LC–MS analysis showed no significant increase in secondary metabolites from *E. grandiflorus* cell suspension cultures from the 5th day up to the 30th day. Furthermore, some secondary metabolites such as elaeocarpenine from the alkaloid group were not detected on the 15th days. Other compounds from the dicarboxylic acid group, such as fumaric acid and succinic acid, were not detected on the 15th and 20th day, respectively (Table 3).

The most abundant compounds identified based on the LC–MS analysis was kaempferol from the flavonoid group. The low concentration of picloram contributed to increased flavonoid biosynthesis and effectively increased secondary metabolic productivity compared to the high dose of synthetic auxin. Furthermore, picloram and 2,4-D have a similar structure and metabolism to natural IAA, but they cannot be degraded and eliminated.[18] Meanwhile, picloram and 2,4-D may regulate metabolism at the cellular level through the exact mechanism of auxin, which is mediated by an auxin-influx carrier or auxin resistant 1/like aux1 (AUX1/LAX) protein.[19,20] Picloram and 2,4-D then influence the phosphorylation of auxin/IAA repressor proteins and trigger the regulation of the associated genes.

Low doses of picloram and 2,4-D were correlated with rate of cell growth. Auxin encourages Aux/IAA complex formation with auxin-to-respond factor (ARF) at low concentrations to suppress the auxin-induced gene expression.[21,22] Gene regulation is not expressed in environments with abundant auxin. At high concentrations, auxin binds to and acts as an adhesive for Aux/IAA proteins to attach to the F-box protein-transport inhibitory respond 1 (TIR1) and mediate degradation.[23,24] This process reduces the amount of Aux/IAA protein in the cytoplasm, which increases the formation of ARF homodimers and the chance to bind to auxin response elements (AuxREs).[25] Furthermore, the protein TIR1 and auxin-related F-box (AFB) proteins are gene-related nuclear receptors regulated by the auxin. However, AFB1-3 has a higher affinity for 2,4-D than TIR1,[20,] and the appearance of 2,4-D at high doses tends to form a complex molecule with TIR1/AFB1-3, which triggers the degradation of Aux/IAA. High amounts of auxin also result in the inhibition of signaling pathways in root differentiation.[24] Therefore, the use of 2,4-D in high doses may reduce the growth rate or the production of secondary metabolites. This is consistent with the common use of 2,4-D, which is applied as a weed inhibitor herbicide.

Its molecular structure similarity to IAA influences the inhibition mechanism of picloram and 2,4-D. The dichlorophenyl ring and two chlorine atoms in the 2,4-D molecule have a similar reactivity to IAA when interacting with TIR1, and the hydrophobic charge of the molecule mimics the characteristics of IAA.[18] For the other mechanism, an AFB structure may be more suitable for binding with picloram but induce the exact same mechanism as 2,4-D.[26] However, low doses of picloram and 2,4-D effectively trigger cell proliferation, organ differentiation, and organ formation by regulating growth mechanisms.[27,28]

The presence of auxin at low concentrations promotes cell cycle progression by regulating the mitosis-related genes expression through several mechanisms. First, it triggers cyclin-dependent kinase complexes' formation by activating catalytic cyclin-dependent kinase-A (CDKA) and D-type cyclins (CYCD).[29] Second, auxin inhibits kinase inhibitory protein (KIP)-related protein (KRP) but activates CDKA/CYCD and phosphorylates retinoblastoma-related protein (RBR). Auxin releases the E2F/DPA complex that promotes the cell cycle transition from the first growth phase (G1) to the synthesis phase (S) and triggers gene expression during interphase.[30]

At the same time, the CDKA/CYCD protein and auxin initiate the cell cycle transition from G1 to S phase.[31] Furthermore, auxin triggers the degradation of S-phase kinase-associated protein 2 (SKP2), thereby activating SKP1-Cullins1-F-box protein (SCF) E3 ubiquitin-protein ligase to degrade E2FC/DPB/RBR repressor protein (inhibitor of the mitotic-related gene during the S phase).[32,33] It also inhibits the degradation of E2FA-B/DPA protein from RBR, thereby triggering the activity of S-phase protein synthesis. Phytohormones, including auxin and cytokines, activate a CDC25-like phosphatase that is involved in the cycle transition from the G2 to the M phase.[34]

The phase transition process in the cell cycle escalates continuous division, increasing cell volume and biomass. By following the concentration of each compound that did not change from the 5th to the 30th day, auxin, carbon, and energy may still be required for growing and building the cell's structural components. According to Dinda et al. (2018),[35] it is relevant that during cell growth, all available resources in the culture media are mainly used for cell division rather than secondary metabolite production. Furthermore, based on the plant biosynthesis mechanism, plant cells mainly produce secondary metabolites from sugar-derivative compounds; such as glyceraldehyde-3 phosphate (G3P).[36] Meanwhile, G3P is converted into sugar during cell growth to produce energy for cytogenesis and structural components such as cellulose.

The secondary metabolite profile is different depending on the tissue or organ type, developmental stage, and environmental condition. More specifically, secondary metabolites such as alkaloids, tannins, and phenolic compounds are generally synthesized to adapt to environmental stress conditions,[37,] including physical and chemical stress or a defense mechanism against pathogens. Even so, the secondary metabolites, especially kaempferol, are pharmacologically necessary for the beneficial drugs. Several studies has proven that kaempferol improves brain tissue healing after injury, prohibits oxidative stress,[38] increases lung cancer apoptosis and autophagy,[39] and prevents various diseases.[40]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 2: The content of various secondary metabolites in the *E. grandiflorus* cell suspension culture on WPM medium with the addition of picloram and 2,4-D

Groups	Components	Concentration (%)					
		PC.1	PC.2	PC.3	2,4-D_1.5	2,4-D_2.5	2,4-D_3.5
Alkaloids	Grandisine	3.80 ± 0.48	3.32 ± 0.51	3.83 ± 0.42	3.40 ± 0.00	3.42 ± 0.91	4.32 ± 0.45
	Elaeokanine C	1.17 ± 0.00	1.19 ± 0.01	1.18 ± 0.05	1.23 ± 0.03	1.17 ± 0.01	1.18 ± 0.00
	Elaeocarpenine	0.65 ± 0.02	0.66 ± 0.02	0.64 ± 0.03	0.67 ± 0.03	0.63 ± 0.01	0.64 ± 0.01
Dicarboxylic Acids	Fumaric acid	0.55 ± 0.53	1.08 ± 0.02	1.06 ± 0.04	1.10 ± 0.05	1.05 ± 0.18	0.87 ± 0.09
	Malic acid	0.95 ± 0.01	0.96 ± 0.02	0.94 ± 0.04	0.98 ± 0.16	1.14 ± 0.20	0.94 ± 0.10
	Succinic acid	n/d	0.83 ± 0.02	0.81 ± 0.03	0.84 ± 0.04	0.80 ± 0.01	0.81 ± 0.00
Flavonoids	Kaempferol	25.07 ± 1.80	23.27 ± 1.48	24.75 ± 1.38	23.36 ± 1.19	24.55 ± 018	24.73 ± 0.09
	Quercetin	6.81 ± 0.11	6.71 ± 0.02	6.73 ± 0.28	7.00 ± 0.33	6.67 ± 0.05	6.72 ± 0.03
	Procyanidin	3.61 ± 0.01	3.60 ± 0.04	3.56 ± 0.15	3.71 ± 0.18	3.54 ± 0.02	3.56 ± 0.01
Phenolic Acids	Epigallocatechin	4.09 ± 0.06	4.03 ± 0.00	4.04 ± 0.17	4.20 ± 0.20	4.01 ± 0.03	4.04 ± 0.02
	Gallic acid	2.93 ± 0.05	2.87 ± 0.02	2.89 ± 0.12	3.01 ± 0.14	2.87 ± 0.02	2.90 ± 0.01
	p-Coumaric acid	1.56 ± 0.01	1.55 ± 0.01	1.54 ± 0.06	1.61 ± 0.08	1.53 ± 0.08	1.45 ± 0.04
Terpenoids	Citronellal	0.91 ± 0.01	0.91 ± 0.02	0.89 ± 0.04	0.93 ± 0.04	0.89 ± 0.01	0.89 ± 0.00
	Citronellol	0.88 ± 0.01	0.89 ± 0.02	0.87 ± 0.04	0.90 ± 0.04	0.86 ± 0.01	0.87 ± 0,00
	Lutein	0.54 ± 0.01	0.53 ± 0.00	0.53 ± 0.02	0.51 ± 0.01	0.52 ± 0.02	0.54 ± 0.01
Vitamins	Ascorbic acid	1.48 ± 0.03	1.45 ± 0.02	1.43 ± 0.03	1.40 ± 0.02	1.43 ± 0.53	0.90 ± 0.43
	Niacin	0.69 ± 0.59	1.27 ± 0.14	1.41 ± 0.06	1.35 ± 0.52	0.83 ± 0.12	0.71 ± 0.33
	α-Tocopherol	0.22 ± 0.00	0.22 ± 0.02	0.24 ± 0.03	0.21 ± 0.00	0.22 ± 0.03	0.24 ± 0.02

Note: n/d = not detected. PC1-3 represents the treatment with 2.5 mg/L, 5 mg/L, and 7.5 mg/L of picloram, respectively. Then 2,4D 1-3 represents the treatment with 1.5 mg/L, 2.5 mg/L and 3.5 mg/L of 2,4-D, respectively.

Table 3: Secondary production in the *E. grandifloras* cell suspension culture on WPM medium with the addition of 2.5 mg/L 2,4-D

Comp. Group	Comp. Name	Days					
		5^{th}	10^{th}	15^{th}	20^{th}	25^{th}	30^{th}
Alkaloids	Grandisine	3.98 ± 0.04	4.01 ± 0.04	3.97 ± 0.17	3.80 ± 0.14	3.67 ± 0.26	3.93 ± 0.03
	Elaeokanine C	1.26 ± 0.02	1.24 ± 0.04	1.20 ± 0.00	1.20 ± 0.02	1.22 ± 0.01	1.21 ± 0.01
	Elaeocarpenine	n/d	0.67 ± 0.01	0.66 ± 0.02	0.65 ± 0.01	0.66 ± 0.01	0.67 ± 0.12
Dicarboxylic acids	Fumaric acid	1.13 ± 0.02	1.11 ± 0.01	n/d	1.08 ± 0.00	1.09 ± 0.00	1.09 ± 0.00
	Malic acid	1.00 ± 0.02	0.98 ± 0.03	0.96 ± 0.01	0.95 ± 0.02	0.97 ± 0.00	0.97 ± 0.00
	Succinic acid	0.87 ± 0.02	0.85 ± 0.02	0.83 ± 0.11	n/d	0.84 ± 0.01	0.84 ± 0.01
Flavonoids	Kaempferol	25.28 ± 0.88	24.40 ± 0.38	24.78 ± 0.33	25.11 ± 1.34	23.77 ± 1.35	25.12 ± 0.37
	Quercetin	7.18 ± 0.12	7.05 ± 0.34	6.72 ± 0.11	6.83 ± 0.12	6.94 ± 0.14	6.81 ± 0.11
	Procyanidin	3.80 ± 0.07	3.74 ± 0.13	3.61 ± 0.01	3.62 ± 0.06	3.68 ± 0.02	3.66 ± 0.02
Phenolic acids	Epigallocatechin	4.31 ± 0.07	4.23 ± 0.19	4.04 ± 0.19	4.10 ± 0.07	4.17 ± 0.07	4.09 ± 0.06
	Gallic acid	3.08 ± 0.05	3.03 ± 0.15	2.87 ± 0.06	2.93 ± 0.05	2.98 ± 0.07	2.91 ± 0.06
	p-Coumaric acid	1.65 ± 0.03	1.62 ± 0.07	1.55 ± 0.01	1.57 ± 0.03	1.59 ± 0.02	1.57 ± 0.02
Terpenoids	Citronellal	0.95 ± 0.02	0.94 ± 0.02	0.91 ± 0.01	0.91 ± 0.02	0.92 ± 0.00	0.93 ± 0.00
	Citronellol	n/d	0.91 ± 0.02	0.89 ± 0.01	0.88 ± 0.02	0.89 ± 0.00	0.90 ± 0.15
	Lutein	0.54 ± 0.01	0.53 ± 0.00	0.53 ± 0.02	0.51 ± 0.01	0.52 ± 0.02	0.54 ± 0.01
Vitamins	Ascorbic acid	1.48 ± 0.03	1.45 ± 0.02	1.43 ± 0.03	1.40 ± 0.02	1.43 ± 0.53	0.90 ± 0.43
	Niacin	0.69 ± 0.59	1.27 ± 0.14	1.41 ± 0.06	1.35 ± 0.52	0.83 ± 0.12	0.71 ± 0.33
	α-Tocopherol	0.22 ± 0.00	0.22 ± 0.02	0.24 ± 0.03	0.21 ± 0.00	0.22 ± 0.03	0.24 ± 0.02

Note: n/d = not detected

Interestingly, secondary metabolites may not directly correlate with the growth and development of plant tissues. Thus, the increase in biomass is not necessarily positively correlated with the number of specific secondary metabolites. Most secondary metabolites are synthesized from precursor compounds produced in the Calvin cycle, glycolysis, or Krebs cycle. The precursors are generally C atom-based organic compounds with added amine groups (NH_x) or pentose sugars. Plants have various sugars, including 3-deoxy-*O*-arabinose-heptulosonate phosphate (DAHP), which are metabolized and reduced to synthesize shikimate.[41] Shikimate is involved in synthesizing phenolic groups, aromatic amino acids (tryptophan, tyrosine, and phenylalanine) and primary metabolites.

We found that the percentage of secondary metabolite production did not change significantly despite increasing the synthetic auxin concentrations. Therefore, the growth effects of picloram and 2,4-D may be constant and stable for *E. grandifloras* cell suspensions after 30 days of culture. This study also proved that the cell suspension culture originating from young leaf petioles of the *E. grandifloras* J. E. Smith plant can produce abundant secondary metabolites. The cell suspension culture may need more time to produce secondary metabolites, or the environmental conditions in the media or incubation room did not encourage the synthesis of secondary metabolites. Therefore, strategies to improve the result may also involve applying environmental stress during cell suspension culture to increase the plant's secondary metabolites. Therefore, future research may investigate biological and ecological pressure in cell suspension culture metabolism and growth regulation.

Conclusion

Picloram and 2,4-D at various concentrations did not increase the secondary metabolite concentration or the type of the compound in *E. grandiflorus* cell suspension culture. Furthermore, the results of LC–MS analysis also did not show any significant changes in the percentage of secondary metabolite profiles from the 5th to the 30th day. Therefore, the energy and organic sources may be allocated to support cell suspension culture for growth instead of secondary metabolite production. At least 92 secondary metabolites were identified, with kaempferols from the flavonoid group as the most abundant bioactive compound. Further study should be conducted by adjusting the synthetic auxin concentration lower than the dose used in this research. Additionally, it may be necessary to improve the treatment by applying stress to the cell suspension cultures to increase the production of secondary metabolites.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

The authors like to thank the Directorate of Research and Community Service, Directorate General of Research and Development Strengthening Ministry of Research, Technology and Higher Education of Indonesian Republic, for funding this research with contract number 44.25.3/UN37/PPK.6.8/2021.

References

1. Rahayu ES, Dewi NK, Bodijantoro FPMH. Profile of Elaeocarpus grandiflorus and Ziziphus mauritiana as identity plants of Salatiga and Tegal towns, Central Java Province, Indonesia. J Phys Conf Ser. 2018; 983(1):012195.
2. Prasannan P, Jeyaram Y, Pandian A, Raju R, Sekar S. A Review on taxonomy, phytochemistry, pharmacology, threats and conservation of *Elaeocarpus* L. (Elaeocarpaceae). Bot Rev. 2020: 86(8): 298–328.
3. HardainiyanS, Nandy BC, KumarK. *Elaeocarpus ganitrus* (Rudraksha): A reservoir plant with their pharmacological effects. Int J Pharm Sci Rev Res. 2015: 34(1):55-64.
4. Bualee C, Ounaroon A, Jeenapongsa R. Antidiabetic and Long-term Effects of *Elaeocarpus grandiflorus*. Naresuan Univ J. 2007; 15(1):17-28.
5. Suparmi S, Widiastuti D, Wesseling S, Rietjens IMCM. Natural occurrence of genotoxic and carcinogenic alkenylbenzenes in Indonesian jamu and evaluation of consumer risks. Food Chem Toxicol. 2018; 118(2):53-67.
6. Ribeiro Filho J, de Sousa Falcao H, Maria Batista L, Maria Barbosa Filho J, Regina Piuvezam M. Effects of Plant Extracts on HIV-1 Protease. Curr HIV Res. 2010; 8(7):531-544.
7. Yue W, Ming QL, Lin B, Rahman K, Zheng CJ, Han T, Qin LP. Medicinal plant cell suspension cultures: Pharmaceutical applications and high-yielding strategies for the desired secondary metabolites. Crit Rev Biotechnol. 2016; 36(2):215-232.
8. Corbin JM, McNulty MJ, Macharoen K, McDonald KA, Nandi S. Technoeconomic analysis of semicontinuous bioreactor production of biopharmaceuticals in transgenic rice cell suspension cultures. Biotechnol Bioeng. 2020; 117(10):3053-3065.
9. Santos RB, Abranches R, Fischer R, Sack M, Holland T. Putting the spotlight back on plant suspension cultures. Front Plant Sci. 2016; 7(3):1-12.
10. Dias MI, Sousa MJ, Alves RC, Ferreira ICFR. Exploring plant tissue culture to improve the production of phenolic compounds: A review. Ind Crops Prod. 2016; 82:9-22.
11. Habibah NA, Moeljopawiro S, Dewi K, Indrianto A. Flavonoid production in callus cultures from mesocarp *Stelechocarpus burahol.* Biosaintifika J Biol Biol Educ. 2016; 8(2):214-221.
12. Habibah NA, Nugrahaningsih WH, Musafa F, Rostriana Y, Mukhtar K, Wijawati N, Anggraito YU. Bioactive compounds from callus culture of *Elaeocarpus grandiflorus*. J Phys Conf Ser. 2020; 1567(3):032055
13. Anggraito YU, Nugrahaningsih WH, Musafa F, Mukhtar K, Wijawati W, Rostriana Y,Safitri Habibah NA. Secondary metabolites in *Elaeocarpus grandiflorus* cell culture in WPM medium with various concentrations of PGR. J Phys Conf Ser. 2020; 1524(1):5-10.
14. Habibah NA, Nugrahaningsih WH, Ulung Anggraito Y, Mukhtar K, Wijayanti N, Mustafa F, Rostriana Y. Effect of growth regulators on cell growth and flavonoid production in cell culture of *Elaecarpus grandiflorus*. IOP Conf Ser Earth Environ Sci. 2019; 391(1):012061
15. Hao G, Du X, Zhao F, Shi R, Wang J. Role of nitric oxide in UV-B-induced activation of PAL and stimulation of flavonoid biosynthesis in *Ginkgo biloba* callus. Plant Cell Tissue Organ Cult. 2009; 97(2):175-185.
16. Habibah NA, Moeljopawiro S, Dewi K, Indrianto A. Flavonoid production, growth and differentiation of *Stelechocarpus burahol* (Bl.) hook. f. and th. cell suspension culture. Pakistan J Biol Sci. 2017; 20(4):197-203.
17. Zaman MAK, Azzeme AM, RamLe IK, Normanshah N, RamLi SN, Shaharuddin NA, Ahmad S, Abdullah SNA. Induction, multiplication, and evaluation of antioxidant activity of *Polyalthia bullata* callus, a woody medicinal plant. Plants. 2020; 9(12):1-21.
18. Anjusha S and Gangaprasad A. Callus culture and in vitro production of anthraquinone in *Gynochthodes umbellata* (L.) Razafim. & B. Bremer (Rubiaceae). Ind Crops Prod. 2017; 95(1):608-614.
19. Gurel E, Yucesan B, Aglic E, Gurel S, Verma SK, Sokmen M, Sokmen A. Regeneration and cardiotonic glycoside production in *Digitalis davisiana* Heywood (*Alanya foxglove*). Plant Cell Tiss Organ Cult. 2011; 104(2):217-225.
20. Song Y. Insight into the mode of action of 2,4-dichlorophenoxyacetic acid (2,4-D) as an herbicide. J Integr Plant Biol. 2014; 56(2):106-113.
21. Swarup R and Péret B. AUX/LAX family of auxin influx

carriers-An overview. Front Plant Sci. 2012; 3(10):1-11.
22. Shen CJ, Bai YH, Wang SK, Zhang SN, Wu YR, Chen M, Jiang DA, Qi YH. Expression profile of PIN, AUX/LAX and PGP auxin transporter gene families in *Sorghum bicolor* under phytohormone and abiotic stress. FEBS J. 2010; 277(14):2954-2969.
23. Yamauchi T, Tanaka A, Inahashi H, Nishizawa NK, Tsutsumi N, Inukai Y, Nakazono M. Fine control of aerenchyma and lateral root development through AUX/IAA- And ARF-dependent auxin signaling. Proc Nat Acad Sci USA. 2019; 116(41):20770-20775.
24. Luo J, Zhou JJ, Zhang JZ. Aux/IAA gene family in plants: Molecular structure, regulation, and function. Int J Mol Sci. 2018; 19(1):1-17.
25. Uchida N, Takahashi K, Iwasaki R, Yamada R, Yoshimura M, Endo TA, Kimura S, Zhang, Nomoto M, Tada Y, Kinoshita T, Itami K, Hagihagra& Keiko U Torii Chemical hijacking of auxin signaling with an engineered auxin-TIR1 pair. Nat Chem Biol. 2018; 14(3):299-305.
26. Fendrych M, Akhmanova M, Merrin J, Glanc M, Hagihara S, Takahashi K, Uchida N Utorii K, FrimL J. Rapid and reversible root growth inhibition by TIR1 auxin signalling. Nat Plants. 2018; 4(7):453-459.
27. Li SB, Xie ZZ, Hu CG, Zhang JZ. A review of auxin response factors (ARFs) in plants. Front Plant Sci. 2016; 7: 1–7.
28. Dayan FE, Duke SO, Grossmann K. Herbicides as Probes in Plant Biology. Weed Sci. 2010; 58(3):340-350.
29. Hasegawa J, Sakamoto T, Fujimoto S, Yamashita T, Suzuki T, Matsunaga S. Auxin decreases chromatin accessibility through the TIR1/AFBs auxin signaling pathway in proliferative cells. Sci Rep. 2018; 8(1):1-12.
30. Silveira SS, Sant'anna-Santos BF, Degenhardt-Goldbach J, Quoirin M. Somatic embryogenesis from mature split seeds of jaboticaba (*Plinia peruviana* (poir) govaerts). Acta Sci - Agron. 2020; 42:1-11.
31. Chandler JW. Auxin response factors. Plant Cell Environ. 2016; 39(5): 1014–28.
32. Dudits D, Cserháti M, Miskolczi P, Horváth G V. The growing family of plant cyclin-dependent kinases with multiple functions in cellular and developmental regulation. Cell Cycle Control Plant Dev. 2007; 32:1-30.
33. Vieira P and Engler J de A. Plant cyclin-dependent kinase inhibitors of the KRP family: Potent inhibitors of root-knot nematode feeding sites in plant roots. Front Plant Sci. 2017; 8(9): 1514-1523
34. Iglesias MJ, Terrile MC, Correa-Aragunde N, Colman SL, Izquierdo-Álvarez A, Fiol DF, París R, Sánchez-López N, Marina A, Villalobos LIAC, Estelle M, Lamattina L, Martínez-Ruiz A, Casalongué CA. Regulation of SCFTIR1/AFBs E3 ligase assembly by S-nitrosylation of Arabidopsis SKP1-like1 impacts on auxin signaling. Redox Biol. 2018; 18(6):200-210.
35. Dindas J, Scherzer S, Roelfsema MRG, Von Meyer K, Müller HM, Al-Rasheid KAS, Palme K, Dietrich P, Becker D, Bennett MJ, Hedrich R. AUX1-mediated root hair auxin influx governs SCFTIR1/AFB-type Ca^{2+} signaling. Nat Commun. 2018; 9(1):1-10
36. Shimotohno A and Umeda M. 5 CDK phosphorylation. In: Inzé D (Eds). Cell cycle control and plant development. New Jersey: Blackwell Publishing Ltd; 2007. 114-138 p.
37. Erb M and Kliebenstein DJ. Plant secondary metabolites as defenses, regulators, and primary metabolites: The blurred functional trichotomy. Plant Physiol. 2020; 184(1):39-52.
38. Pachauri S, Chatterjee S, Kumar V, Mukherjee PK. A dedicated glyceraldehyde-3-phosphate dehydrogenase is involved in the biosynthesis of volatile sesquiterpenes in *Trichoderma virens*—evidence for the role of a fungal GAPDH in secondary metabolism. Curr Genet. 2019; 65(1): 243-52.
39. Caser M, Chitarra W, D'Angiolillo F, Perrone I, Demasi S, Lovisolo C, Pistellie L, Pistelli L, Scariota V. Drought stress adaptation modulates plant secondary metabolite production in *Salvia dolomitica* Codd. Ind Crops Prod. 2019; 129(6): 85-96.
40. Chitturi J, Santhakumar V, Kannurpatti SS. Beneficial effects of kaempferol after developmental traumatic brain injury is through protection of mitochondrial function, oxidative metabolism, and neural viability. J Neurotrauma. 2019; 36(8):1264-1278.
41. Han X, Liu CF, Gao N, Zhao J, Xu J. Kaempferol suppresses proliferation but increases apoptosis and autophagy by up-regulating microRNA-340 in human lung cancer cells. Biomed Pharmacother. 2018; 108(826):809-816.
42. Ren J, Lu Y, Qian Y, Chen B, Wu T, Ji G. Recent progress regarding kaempferol for the treatment of various diseases (Review). Exp Ther Med. 2019; 18(8):2759-2576.
43. Zabalza A, Orcaray L, Fernández-Escalada M, Zulet-González A, Royuela M. The pattern of shikimate pathway and phenylpropanoids after inhibition by glyphosate or quinate feeding in pea roots. Pestic Biochem Physiol. 2017; 141(9):96-102.