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Profile of Flavonoid and Antioxidant Activity in Cell Suspension Culture of *Elaeocarpus grandiflorus*

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Abstract. Cell suspension cultures of *Elaeocarpus grandiflorus* produce flavonoids and various secondary metabolites. Flavonoid profile and antioxidant activity of the suspension culture extract of *E. grandiflorus* cells have not been studied. Therefore, this research aimed to analyze the antioxidant activity and flavonoid profile of the cell suspension culture extract of *E. grandiflorus*. Cell suspension cultures of *E. grandiflorus* were produced from leaf stalk callus that grew on WPM medium with a growth regulator 2.5 ppm 2,4-D. Cells were harvested at 30 days old and then extracted for profile analysis of bioactive compounds using the LC-MS method. The antioxidant activity analysis was conceded out using the DPPH method. The results showed that there were 32 types of flavonoids, of which 11 compounds had a concentration of more than 1% of the total bioactive compounds and had the potential to have antioxidant activity. The analysis results also showed that flavonoids type, composition, and antioxidant activity were not significantly different between the ages of *E. grandiflorus* cell suspension cultures. In addition, there was no correlation between flavonoid concentration and antioxidant activity. This study enriches information about secondary metabolites production in the *E. grandiflorus* cell culture which is still very rarely studied because of its rarity. The study also provides scientific basis evident for *E. grandiflorus* cell culture as source of potential antioxidant.

Key words: antioxidant, cell suspension cultures, *E. grandiflorus*, flavonoid, LC-MS

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INTRODUCTION

Cell culture is widely used for secondary metabolites production. Several studies showed bioactive compounds such as andrographolide produced in *Andrographis paniculata* cell culture (Habibah, 2009) and phenolic acids in *Petiveria alliacea* cell suspension culture induced with at 2,4-D (Castellar et al., 2011). Furthermore, potential bioactive compounds such as naphtodianthrones and phenylpropanoids can also be produced through callus culture and *Hypericum perforatum* L. cell suspension. (Gadzovska et al., 2013). In addition, bioactive compounds that have therapeutic potential, such as flavonoids, can be extracted from the cell culture of *Stelechocarpus burahol* (Habibah et al., 2017), while flavonoids and phenolic acids from *Elaeocarpus grandiflorus* (Anggraito et al., 2020; Habibah et al., 2019).

Several studies have shown that flavonoids that have antioxidant activity, including naringenin (Ferreyra et al., 2012; Ghofrani et al., 2015), quercetin (Kumar & Pandey, 2013), and rutin. (Herlina et al., 2018; Tan et al., 2010). Several potential therapeutic properties of the bioactive compounds found in *Elaeocarpus* genera are antiviral activity against influenza (Dao et al., 2019), ability to inhibit *E. coli* growth (Savitri et al., 2020) and antidiabetic activity (Bualee et al., 2007). Furthermore, all parts of *Elaeocarpus* plant organs contain bioactive compounds such as tannin and geraniin, and 3, 4, 5-trimethoxy geraniin in leaf (Shah et al., 2011).

The cell growth and secondary metabolites during *in-vitro* culture are influenced by various factors, including stress, concentrations of exogenous growth regulators (Amoo & van Staden, 2013), and stages of tissue development (Balasubramanya et al., 2012). Cells in cell cultures grow with a particular pattern depending on the species and also the medium used. Some cases, such as cell suspension culture of *Capsicum annuum*, grew and multiplied faster for doubling time and reached 0.72 days per replication (Suthar & Shah, 2015), while the cell suspension of *Petiveria alliacea* entered an exponential phase after four weeks. Furthermore, cell suspension culture induction using growth factors such as picloram or 2,4-D performs various growth patterns and periods of cell division (Castellar et al., 2011). Growth kinetics of *Centella asiatica* cell suspension culture on medium with the addition of 2.0 mg/L 2,4-D and 1 mg/L kinetin indicates an absence of lag phase from wet and dry weight observations. Furthermore, the pattern of secondary metabolite production in cell

culture is also influenced by the culture period. The study of *Phytophthora europaea* cells' suspended culture induced by 2,4-D shows maximum cell growth and high plumbagin production on day 18th (Beigmohamadi et al., 2019).

Based on the explanation, it is necessary to conduct further studies on the production of bioactive compounds in the suspension culture of *Elaeocarpus grandilforus* cells induced by 2, 4-D. This is an effort to find the proper technique and procedure to increase the production efficiency of bioactive compounds in *E. grandilforus* through cell suspension culture. Therefore, this study aimed to analyze the flavonoid profile and antioxidant activity of the suspension culture extract of *E. grandiflorus* cells.

METHOD

This research was an experimental study using *E. grandiflorus* cell suspension culture induced with a single dose of 2, 4-D for 30 days. This study analyzed the flavonoid content of 5, 10, 15, 20, 25 and 30 days-old of *E. grandiflorus* cell suspension culture.

Plants culture preparation

The two-year-old *Elaeocarpus grandiflorus*'s leaf stalk was collected and processed in the Laboratory of Plant Tissue Culture of the Universitas Negeri Semarang, aseptically. The leaf stalk was sterilized using bactericide and fungicide followed by 5.25% NaClO bleach solution. A woody plant medium (WPM) was used as a growth medium and 2.5 ppm of 2,4-D (Sigma Aldric, Darmstadt, Germany) as a growth regulator.

Cell culture induction

The cell culture medium was made using woody-plant medium (WPM) stock dissolved in distilled water by following manufacture procedure. The prepared medium was supplemented with 2.5 ppm 2, 4-D, and added with distilled water to get the required volume. To enrich the nutrition, a 3% sucrose from total solution was added homogenously. The solution was then added with HCl or NAOH until the pH reached 5.8. After medium preparation was complete, approximately 20 ml of the medium was poured into 100 ml Erlenmeyer flasks and covered using sterile cap. The solution in Erlenmeyer flasks were sterilized using autoclave chamber with pressures between 1.1-1.5 kg cm-^{2} and temperature was settled at 121^{o}C, for 20 minutes.

The calluses were well cared for 5-months before ready for cell culture induction. After grew properly, 1 g of callus was transferred into a 100 ml Erlenmeyer containing 20 ml of WPM and shaken at a speed of 120 rpm for next cell suspension culture formation steps. The cell suspension culture was harvested and processed every five days until the 30th day. The harvested cell cultures were filtered, measured, and weighted, then dried in the oven at 60°C for 48 hours.

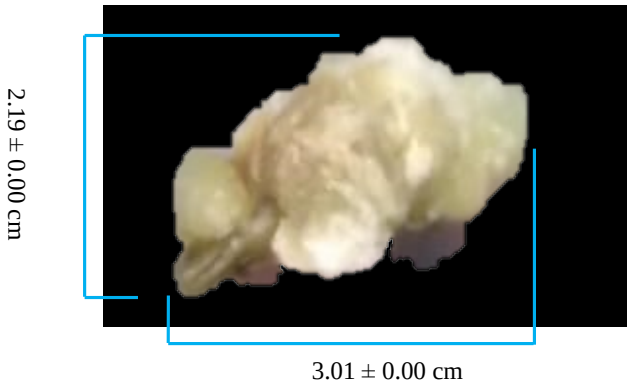

Figure 1. Callus from petiole of *E. grandiflorus* used for suspension culture induction.

Flavonoid extraction

Flavonoids were extracted from the suspension culture of *E. grandiflorus* by following the procedure from Hao et al., (2009). The dry cells were ground using mortar and pestle to make a fine powder, then soaked in methanol-1% HCl solution (v/v) and added with 2 N HCl (v/v). The processed extract was rested and incubated at 90 °C, for one hour, then dried and resuspended in methanol for antioxidant activity and LC-MS analysis.

Antioxidant activity analysis

Five mg of solid DPPH was dissolved into 100 ml of methanol to make DPPH stock solution. A control solution was prepared by mixing 50 ppm DPPH stock solution with 2 ml of methanol and homogenized by pipetting. Two ml of the samples were added with 2 ml DPPH stock solution and incubated at 27 ℃, for 30 minutes. The positive reaction of the DPPH activity was performed by the changing color, it indicated that the samples were ready for the antioxidant activity measurement process. Antioxidant activity was measured using the UV-Vis spectrophotometer absorbance values at a wavelength of 517 nm and conducted for three times repetition.

LC-MS analysis

The obtained extract was dissolved in methanol solvent to reach a concentration below 100 ppm using pipetting technique to obtain a homogeneous solution. Sample's pellet and supernatant was separated using centrifugation at 8000 rpm for 10 minutes. After separation process, the supernatant was collected for protein precipitation was processed. Two ml of the supernatant was put into a centrifuge

tube, added with 3 ml of acetonitrile acidified with 0.2 % formic acid, and centrifuged at 8000 rpm for 30 seconds. The supernatant was used for the purification process by the Solid Phase Extraction (SPE) method. The solution was filtered with a 0.45 m cellulose acetate filter membrane, and degassing was carried out. The solution was ready to be used for injection into the LC-MS (liquid chromatography-mass spectrometry). The LC-MS apparatus model used was Shimadzu LCMS – 8040 LC/MS with Column Shimadzu Shim Pack FC-ODS (2 mm x 150 mm, 3 m). Injection volume of 1 L, capillary voltage of 3.0 kV, column temperature of 35°C, isocratic mobile phase mode, flow rate of 0.5 ml/min, mobile phase of methanol 90% with water, MS focused ion mode Io type [M]+, collision energy of 5.0 V, desolvation gas flow of 60 ml/hr at temperature of 350 °C, fragmentation method of low energy CID, ESI ionization, scanning of 0.6 sec/scan (Mz: 10-1000), source temperature of 100 °C, and run time of 80 minutes.

RESULT AND DISCUSSION

Several studies reported that different part of *Elaeocarpus* species has various concentration of flavonoid (Shah et al., 2011), specifically, the flavonoid content that was extracted using 95% ethanol from *E. serratus* reaching up to 92.35 ± 2.85 mg of total flavonoids content (TFC)/ g dry weight (DW) (Chen & Yang, 2020), then reaching 273.58 ± 2.14 mg TFC/ g DW in *E. mastersii* (Okselni et al., 2018), and 37.5 mg TFC/g DW in *E. sphaericus* (Deepika et al., 2018). However, the exact flavonoid concentration in *E. grandiflorus* leaves is still unknown properly

In this research, The LC-MS analysis showed that the cell suspension extract of *E. grandiflorus* contained about 87 to 90 types of secondary metabolites, including the various member of alkaloids, dicarboxylic acids, flavonoids, phenolic acids, tannins, phytosterols, terpenoids, vitamins, and several other combinations (Table 1).

Table 1. LC-MS data of *E. grandiflorus* cell suspension extracts

RT (min)	Compound assignment*	Molecular Formula	Culture Ages (days)					
			5	10	15	20	25	30
1.238	Fumaric acid	$C_4H_4O_4$	√	√	-	√	√	√
1.289	Benzoic acid	$C_7H_6O_2$	√	√	√	√	√	√
1.473	Malic acid	$C_4H_6O_5$	√	√	√	√	√	√
1.670	Ethyl cinnamate	$C_{11}H_{12}O_2$	√	√	√	√	√	√
1.839	P-Coumaric acid	$C_9H_8O_3$	√	√	√	√	√	√
3.042	Gallic acid	$C_7H_6O_5$	√	√	√	√	√	√
5.043	Ferulic acid	$C_{10}H_{10}O_4$	√	√	√	√	√	√
5.826	Elaeokanine C	$C_{12}H_{21}NO_2$	√	√	√	√	√	√
10.332	Kaempferol	$C_{15}H_{10}O_6$	√	√	√	√	√	√
10.500	Epicatechin	$C_{15}H_{14}O_6$	√	√	√	√	√	√
11.427	Quercetin	$C_{15}H_{10}O_7$	√	√	√	√	√	√
11.503	Epigallocatechin	$C_{15}H_{14}O_7$	√	√	√	√	√	√
12.421	Chlorogenic acid	$C_{16}H_{18}O_9$	√	√	√	√	√	√
20.063	Kaempferol-4'-rhamnoside	$C_{21}H_{20}O_{10}$	√	√	√	√	√	√
21.385	Isovitexin	$C_{21}H_{20}O_{10}$	√	√	√	√	√	√
21.267	Vitexin	$C_{21}H_{20}O_{10}$	-	√	√	√	√	√
21.429	Kaempferol-3-O-rhamnoside	$C_{21}H_{20}O_{10}$	√	√	√	√	√	√
22.172	Orientin	$C_{21}H_{20}O_{11}$	√	√	√	√	√	√
22.174	Quercetin-3-O-rhamnoside	$C_{21}H_{20}O_{11}$	√	√	√	√	√	√
22.183	Epicatechin gallate	$C_{22}H_{18}O_{10}$	√	√	√	-	√	√
22.619	Isoorientin	$C_{21}H_{20}O_{11}$	√	-	√	√	√	-
22.623	Kaempferol-3-O-D-glucoside	$C_{21}H_{20}O_{11}$	√	√	√	√	√	√
22.628	Luteolin-7-glucoside	$C_{21}H_{20}O_{11}$	√	√	√	√	√	√
23.705	Epi gallocatechin-3-O-gallate	$C_{22}H_{18}O_{11}$	√	√	√	√	√	√
23.954	Kaempferol 3-glucuronide	$C_{21}H_{18}O_{12}$	√	√	√	√	√	√
24.001	Quercetin 3-glucoside	$C_{21}H_{19}O_{12}$	√	√	√	√	√	√
24.020	Hyperoside	$C_{21}H_{20}O_{12}$	√	-	√	√	√	√
24.032	Isoquercetin	$C_{21}H_{20}O_{12}$	-	√	√	√	√	√
24.768	Kaempferol 3-(2"acetylrhamnoside)	$C_{23}H_{22}O_{11}$	√	√	√	√	-	√

RT (min)	Compound assignment*	Molecular Formula	Culture Ages (days)					
			5	10	15	20	25	30
24.770	Kaempferol 3-(3"-acetylrhamnoside)	$C_{23}H_{22}O_{11}$	√	-	√	√	√	√
24.773	Kaempferol 3-(4"-acetylrhamnoside)	$C_{23}H_{22}O_{11}$	√	√	√	√	√	√
25.835	Querciturone	$C_{21}H_{18}O_{13}$	√	√	√	√	√	√
28.206	Kaempferol 3-(2",4'-diacetylrhamnoside)	$C_{25}H_{24}O_{12}$	√	√	√	√	√	√
28.208	Kaempferol 3-(3",4"diacetylrhamnoside)	$C_{25}H_{24}O_{12}$	√	√	√	√	√	√
33.429	Procyanidin A1	$C_{30}H_{24}O_{12}$	√	√	√	√	√	√
33.496	Procyanidin B1	$C_{30}H_{26}O_{12}$	√	√	√	√	√	√
33.498	Procyanidin B2	$C_{30}H_{26}O_{12}$	√	√	√	√	√	√
33.563	Naringin	$C_{27}H_{32}O_{14}$	√	√	√	√	√	√
34.003	Kaempferol-7-rhamnoside-4'-glucoside	$C_{27}H_{30}O_{15}$	√	√	√	√	√	√
34.016	Kaempferol-3-(5"-feruloylapioside)	$C_{30}H_{26}O_{13}$	√	√	√	√	√	√
35.508	Kaempferol-3-(6"caffeoylglucoside)	$C_{30}H_{26}O_{14}$	√	√	√	√	√	√
35.517	Rutin	$C_{27}H_{30}O_{16}$	√	√	√	√	√	√
62.166	Geraniin	$C_{41}H_{28}O_{27}$	√	√	√	√	√	√

Note: *The components listed are only those with a concentration of more than 1%.

Based on the identification results, each culture age mostly produced secondary metabolites with no significant difference in type and composition. However, some compounds were not detected in several periods or cannot be detected because of insufficient quantities. It contradicts Hernández-Altamirano et al. (2020) research, which stated that encecalin produced in *Helianthella quinquenervis* cultures varied greatly depending on the growth curve. Meanwhile, in this study, observation on the dried cell culture sample indicates that cell growth did not reach an exponential phase even for up to 30 days. Slow cell growth during suspension culture may affect the bioactive productivity and compounds profile because most of the cell resource is used for growing. It is in line with the Wijawati et al. (2019) research, which explained that *E. grandiflorus* callus takes 10 to 22 days to produce and harvests after five months. The study only studied the type and composition of secondary metabolites of *E. grandiflorus* culture cell extract but has not studied the concentration of each secondary metabolite. It is likely that secondary metabolite concentration of each culture age are different, which will be studied in future studies.

Furthermore, the most abundant compound was flavonoid, with more than 50% of total secondary metabolites consisting of 32 compounds. The highest flavonoid concentration is kaempferol group compounds, with composition reaching more than 48% of total flavonoid (Figure 2).

Eleven flavonoid compounds detected in cell suspension cultures showed no significant changes in concentration until day 30th, except for vitexin and isoorientin. Vitexin was undetectable in the first five days, then measured after. Meanwhile, isoorientin was not detected on the 10th and 30th days of observation. Meanwhile, kaempferol was the most abundant flavonoid compound in all callus ages, followed by quercetin, procyanidin, luteolin, and naringin.

Flavonoids consists of more than 6500 molecules based upon a 15-carbon skeleton. The molecules structure are formed from 2-phenylbenzopyranone as a core chain, which oxygen cyclized to three-carbon bridge between phenyl groups. Therefore, flavonoids have been recognized as influential and most widespread secondary plant metabolites, with marked antioxidant properties (Corradini et al., 2011). Various studies have shown that each type of flavonoid has varying activity levels and therapeutic potential, as described in Table 2.

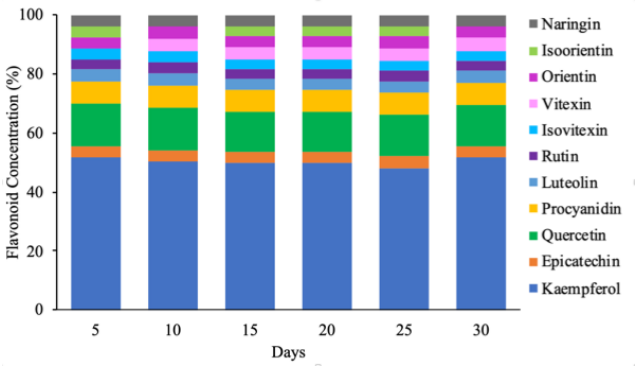

Figure 2. Flavonoid main composition of *E. grandiflorus* cell suspension extracts

Table 2. Type of flavonoid and bioactivity

Flavonoid	Activity	Ref.
Kaempferol	Analgesic component, antiallergic compound, antibacterial activity, inhibit cancer growth, antidiabetic activity, oxidant scavenger, anti-inflammatory compound, enhance calcium absorption, anxiolytic agent, act as like as estrogen, protect cardiovascular stability and neuron,	Calderón-Montaño et al., (2011)
Epicatechin	Inhibit cancer growth, anti-inflammatory compound, inhibit bacteria metabolism.	Tvrda et al., (2019)
Quercetin	Oxidant scavenger, inhibit cancer growth, protect hepatocyte from destructive agent, anti-inflammatory effect, and enhance immune system during infection.	Huang et al., (2016; Kumar & Pandey, 2013)
Procyanidin	cardiovascular diseases suppressor, antidiabetic agent, inhibit metastasis step, anti-inflammatory compound, oxidant scavenger, anti-atherosclerosis agent, increase plaque stability, and reduce vascular calcification.	Liang et al., (2021)
Luteolin	antioxidant, relaxant in vascular permeability, and anti-inflammatory.	Seelinger et al., (2008)
Rutin	Provide scavenging activity against free radicals, hepatoprotective activity, antifungal activity, anti-inflammatory compound, anticancer effect, inhibit leukemia, strengthen blood vessel	Enogieru et al., (2018); Yang et al., (2008)
Isovitexin	antioxidant.	Chowjarean et al., (2019)
Vitexin	Anti-inflammatory compound, cardioprotective agent, prohibit metastasis, antinociceptive agent, anticonvulsant activity, memory enhancing potential, and anti-diabetic activities.	Babaei et al., (2020)
Orientin	Anti-adipogenesis, antiaging, antiallergic, antibacterial, anticancer, antidepressant, antidiabetic, antinociceptive effects, antioxidant, anti-osteoporotic, antiviral, anxiolytic, estrogenic/antiestrogenic, cardioprotective, neuroprotective, and vasodilatation stimulus.	Lam et al., (2016)
Isoorientin	Scavenging free radical.	Yuan et al., (2016)
Naringin	Eliminate radical oxygen species and potent as a drug of *Alzheimer's* disease.	Ferreyra et al., (2012); Ghofrani et al., (2015)

Antioxidant activity analysis on flavonoids showed no difference at all ages of culture but may potentially be applicable for antioxidant compounds. It was supported by the results of DPPH analysis, which showed that antioxidant activity was more than 30% (Table 3). Therefore, further study is needed to evaluate the function and potential of each flavonoid compound in the cell suspension culture of *E. grandiflorus*. The scavenging activity depends on the hydroxyflavone structure, which is neighboring hydroxyl groups. It may showed higher antioxidative activities than flavonoid compounds with separated hydroxyl (Ashraf et al., 2020). Therefore, the ortho position of dihydroxyl groups is one of the structural conditions of hydroxyflavone for the good scavenging effect (Dai et al., 2017; Samsonowicz et al., 2017).

Antioxidant activity at all culture ages showed a constant value but tended to decrease until day 30. This was probably due to the low growth of cell suspension cultures, which impacted on the antioxidant activity. Even though, based on the analysis, there is no significant correlation between antioxidant activity and total flavonoid content of *E. grandiflorus* cell suspension extracts (Table 4)

Table 3. Antioxidant activity of *E. grandiflorus* cell suspension extracts

Age culture (days)	Antioxidant activity (%)
5	50.28±5.84
10	56.19±7.82
15	54.27±3.21
20	57.97±3.49
25	54,23±4,.54
30	39.77±16.09

Table 4. Correlation value between total flavonoid content and antioxidant activity of the *E. grandiflorus* cell suspension extracts

Variable		TPC
Antioxidant activity	Pearson Correlation	-0.805
	Sig. (2-tailed)	0.053

Note: TFC = total phenolic content, significant level is ≤ 0.050 at confident level = 95%

Antioxidant activity is not only influenced by flavonoids but also various bioactive compounds, including tannins, polyphenols, carotenoids, and others (Zaman et al., 2020). Therefore, it shows that flavonoids contents may not represent a significant relationship with antioxidant activity. On the other side, the antioxidant activity and flavonoids content indicate stagnant production of the antioxidant-potential bioactive compounds, even though the cell suspension was cultured for up to 30 days. Today, *E. grandiflorus* recognized as a rare species, and exploitation of the species is restricted (Rahayu et al., 2018). However, the organ and tissue of this species contains rich secondary metabolites that used as antidiabetic. Therefore, this study enriches the information and proves that *E. grandiflorus* cell culture contains antioxidants from secondary metabolites which is still very rarely studied because of its rarity. The study also provides scientific basis evident for *E. grandiflorus* cell culture application as source of potential antioxidant.

CONCLUSION

E. grandiflorus cell suspension culture can produce various kinds of flavonoids that have antioxidant activity. Eleven main flavonoids contained in the cell suspension extract of *E. grandiflorus* are flavonoids that have antioxidant activity. Flavonoids produced at each age are not much different, as well as their antioxidant activity. There are no significant correlation between antioxidant activity and flavonoid concentration.

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