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 2,4-D (Castellar et al., 2011). Furthermore, potential bioactive compounds such as naphthodianthrones 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 Stelchocarpus 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 (Ferreira 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 (Saviti 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 (Balasubramanay 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 grandiflorus* 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. grandiflorus* 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 homogeneously. 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°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 °C, 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).

| RT (min) | Compound assignment* | Molecular Formula | Culture Ages (days) |
|----------|----------------------|-------------------|--------------------|
| 1.238    | Fumaric acid         | C₄H₄O₄            | -      |
| 1.289    | Benzoic acid         | C₆H₅O₂            | -      |
| 1.473    | Malic acid           | C₄H₆O₅            | -      |
| 1.670    | Ethyl cinnamate      | C₁₁H₁₂O₄          | -      |
| 1.839    | P-Coumaric acid      | C₆H₅O₃            | -      |
| 3.042    | Gallic acid          | C₆H₄O₅            | -      |
| 5.043    | Ferulic acid         | C₁₀H₁₀O₄          | -      |
| 5.826    | Elaeokanine C        | C₁₂H₁₂NO₂         | -      |
| 10.332   | Kaempferol           | C₁₃H₁₀O₆          | -      |
| 10.500   | Epicatechin          | C₁₃H₁₄O₄          | -      |
| 11.427   | Quercetin            | C₁₃H₁₀O₇          | -      |
| 11.503   | Epigallocatechin     | C₁₃H₁₄O₇          | -      |
| 12.421   | Chlorogenic acid     | C₁₆H₁₈O₈          | -      |
| 20.063   | Kaempferol-4’-rhamnoside| C₂₁H₂₀O₁₀       | -      |
| 21.385   | Ivovitin             | C₂₁H₂₀O₁₀         | -      |
| 21.267   | Vitexin              | C₂₁H₂₀O₁₀         | -      |
| 21.429   | Kaempferol-3-O-rhamnoside| C₂₁H₂₀O₁₀    | -      |
| 22.172   | Orientin             | C₂₁H₂₀O₁₁         | -      |
| 22.174   | Quercetin-3-O-rhamnoside| C₂₁H₂₀O₁₁       | -      |
| 22.183   | Epicatechin gallate  | C₂₁H₁₈O₁₀         | -      |
| 22.619   | Isoorientin          | C₂₁H₂₀O₁₁         | -      |
| 22.623   | Kaempferol-3-O-D-glucoside| C₂₁H₂₀O₁₁     | -      |
| 22.628   | Luteolin-7-glucoside| C₂₁H₂₀O₁₁         | -      |
| 23.705   | Epi galocatechin-3-O-gallate| C₂₁H₁₈O₁₁   | -      |
| 23.954   | Kaempferol 3-gluconuride| C₂₁H₁₈O₁₂        | -      |
| 24.001   | Quercetin 3-gluconuride| C₂₁H₁₉O₁₂        | -      |
| 24.020   | Hyperoside           | C₂₁H₂₀O₁₂         | -      |
| 24.032   | Isoquercetin         | C₂₁H₂₀O₁₂         | -      |
| 24.768   | Kaempferol 3-(2"acetyl)rhamnoside)| C₂₂H₂₂O₁₁     | -      |
The identification results showed for *Helianthella quinquenervis*. It is lin-nidin, luteor vitexin and, ample indicates that ps. 0, which is detected in th th. Concentr suspensions showed no significant changes in flavonoid metabolites flavonoid metabolites in cell suspension cultures showed no significant changes in flavonoid concentration. Furthermore, in this study, the culture age comprised of secondary metabolites takes 10 to 22 days growing profile is different with one another. Meanwhile, in this study, cell cultures varied greatly depending on the growth profile.

### Table 2

| Compound Assignment          | Molecular Formula | Culture Ages (days) |
|-----------------------------|-------------------|---------------------|
| Kaempferol 3- (3''-acetyl) | C_{22}H_{22}O_{11} | 5 10 15 20 25 30   |
| Kaempferol 3- (4''-acetyl) | C_{22}H_{22}O_{11} |                     |
| Quercitrone                 | C_{21}H_{18}O_{13} |                     |
| Kaempferol 3- (2''4''-dia   | C_{22}H_{24}O_{12} |                     |
| Kaempferol 3- (3''4''-dia    | C_{22}H_{24}O_{12} |                     |
| Procyanidin B1              | C_{30}H_{26}O_{12} |                     |
| Procyanidin B2              | C_{30}H_{26}O_{12} |                     |
| Naringin                    | C_{27}H_{12}O_{14} |                     |
| Kaempferol-7-rhamnoside4'-g | C_{27}H_{30}O_{15} |                     |
| Kaempferol-3- (5''-feru      | C_{30}H_{26}O_{13} |                     |
| Kaempferol-3- (6''caffeoyl   | C_{30}H_{26}O_{14} |                     |
| Rutin                       | C_{27}H_{30}O_{16} |                     |
| Geraniin                    | C_{41}H_{38}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 consist 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 growth, anti-diabetic activity, antioxidant 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. | Tyrda 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, antiosteoporotic, 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        | Pearson Correlation   | Sig. (2-tailed) |
|-----------------|-----------------------|-----------------|
| Antioxidant activity | -0.805                | 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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