Journal of Integrated and Advanced Engineering (JIAE)
Vol. 2, No. 1, March 2022: 33-44
http://asasijournal.id/index.php/jiae
http://doi.org/10.51662/jiae.v2i1.37

Analysis of Plant Growth and Gallic Acid Content for Cavendish Banana (*Musa acuminata*) Shoot Culture with Bubble Column Bioreactor

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Abstract
Cavendish banana (Musa acuminata) is one of the most important fruits in the world. Cavendish shoots tissue culture using bubble column bioreactor can be a solution to produce high yielding plantlet and gallic acid due to the aeration with minimum shear stress. In this study, the average growth rate, presence of gallic acid, and antioxidant activity (IC50) in the bubble column bioreactor (200 mL capacity) with the aeration rates of 1 mL/s and 2 mL/s using Murashige & Skoog half-strength liquid medium supplemented with 0.5 ppm gibberellic acid will be analyzed. The aeration system used was atmospheric air. The leaves and stems were extracted by maceration using 96% ethanol solvent (1:10 (w/v)). A qualitative phenolic test with $FeCl_3$, thin layer chromatography, and antioxidant test with 2,2-diphenyl-1-picrylhydrazyl was carried out. The average growth rate in the bioreactor were 0.22 ± 0.001 g/day (1 mL/s) and 0.21 ± 0.001 g/day (2 mL/s). All the leaf and stem extracts showed positive results for the phenolic test, but the presence of gallic acid could not be detected clearly by thin-layer chromatography. The IC50 values in aeration rates of 1 mL/s and 2 mL/s of the leaves were 41.35 and 79.54 µg/mL, respectively, while the stems were 51.87 and 104.94 µg/mL, respectively. It could be concluded that the growth of the banana plantlet and the production of antioxidants in the bubble column bioreactor was higher in aeration rate of 1 mL/s than 2 mL/s.

Keywords:
Bubble Column Bioreactor;
Cavendish Banana;
Gallic Acid;
Tissue Culture;

Article History:
Received: January 25, 2022
Revised: March 12, 2022
Accepted: March 17, 2022
Published: March 30, 2022

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INTRODUCTION

Cavendish banana (*Musa acuminata*) is one of the most important commercial fruits in the world. This banana is very popular and has become the most widely traded globally, including in Indonesia and Malaysia [1]. Based on Statistics Indonesia (BPS), banana production in Indonesia generally reached 7,280,658 tons in 2019, and it took as the first position for the fruits category [2]. Cavendish banana belongs to the Musaceae family with AAA triploid [3]. The fruit of Cavendish banana has a distinctive shape and taste as well as contains complete nutrients, such as carbohydrates, fiber, potassium, calcium, manganese, and vitamins, so it is very popular with people in various regions [4]. Apart from the fruit, other parts of the banana plant, particularly the leaves and stems (pseudo-stems), have much medicinal use [5]. In addition, Cavendish banana plants can also widely adapt to various conditions in tropics and subtropics [6].

The production of superior seeds using tissue culture can increase productivity and so to overcome the high demand for Cavendish bananas [7]. Tissue culture is an *in vitro* plant propagation that is carried out under controlled conditions by utilizing tissue and the totipotency of plants to produce large numbers of plant clones in a short time [8, 9, 10]. Compared to conventional methods, the advantages of tissue culture techniques include similarity of genetic characteristics as mother plants, disease-free, faster growth, and faster

maturation process [11]. On the other hand, tissue culture is also very beneficial for production of certain metabolites, because propagation can be adapted to the tissue with the highest content of the desired metabolite so that the extraction process and metabolite acquisition will be more efficient [11].

The use of bioreactors in tissue culture can also optimize the growth process and accumulation of secondary plant metabolites. One of the potential bioreactors to be developed is the bubble column bioreactor. A bubble column bioreactor is a multiphase cylindrical reactor with a submerged culture system equipped with a bubble gas spout into the liquid system or liquid-solid suspension [12]. The bubbles are used to mix, circulate, and aerate nutrients in medium culture with minimal shear stress not to damage the culture [13]. This causes high gas transfer and nutrient transfer rates [14]. In addition, the use of liquid media in bioreactors also has several advantages, such as renewing the air during cultivation and higher plantlet growth rates and lower production costs compared to solid media [15].

The Cavendish banana plant can also be developed through the biorefinery concept by utilizing its metabolite content. Parts of the banana plant other than fruit, such as leaves and stems, which are often only used as low-value products or simply thrown away, contain polyphenol compounds in the form of gallic acid, which has the pharmacological and economic potential [16]. Gallic acid (*3,4,5-trilhydroxybenzoic acid*) is a plant polyphenol found in tea, berries, grapes, and other fruits that is cytotoxic for many types of tumor cells and is used as a chemoprevention drug in cancer treatment [17]. Gallic acid affects several pharmacological and biochemical pathways, making it known as a strong antioxidant, anti-inflammatory, antimutagenic and anticancer compound [17]. Sagrin & Chong [18] reported that banana leaves have a quite high gallic acid content, which is around 2.7% (w/w). In banana stems and humps, a small number of gallic acid and other phenolic compounds have also been found [19, 20, 21]. Therefore, banana plants offer a great opportunity as an affordable source for producing bioactive compounds.

This study aims to compare the plant growth, the presence of gallic acid, and the antioxidant activity of Cavendish banana shoots (*Musa acuminata*) in bubble column bioreactor at various aeration rates of 1 mL/s dan 2 mL/s.

METHOD

Materials

In this study, Cavendish banana explants were obtained from the School of Life Sciences and Technology inventory, Bandung Institute of Technology, Ganesha Campus, with the characteristic of about 3-4 leaves and was approximately one month after the initiation process. The chemicals used in this study were Murashige & Skoog (MS) medium (PhytoTech LABS), sucrose, *Gibberellic Acid* (GA3) (HIMEDIA), ethanol (Merck, 96%), $FeCl_3$ (Smart-Lab), and *2,2-diphenyl-1-picrylhydrazyl* (DPPH) (Smart-Lab).

Medium Preparation

The pH condition in each medium was set at 5.8. Then, the medium was sterilized by autoclaving at 121°C for 15 minutes.

Cavendish Banana Shoot Subculture and Acclimatization

The method used has been optimised in the School of Life Sciences and Technology laboratory for four years. The medium used in the subculture process was a solid growth medium consisting of full-strength Murashige & Skoog (MS) medium, 30 g/L sucrose, 1 ppm

Gibberellic Acid (GA3), and 8 g/L agar. Subcultures were performed three times within six weeks. First, the newly formed shoots were ready to be acclimatised in a liquid medium consisting of half-strength MS and sucrose 20 g/L without hormones. Banana cultures were then incubated at room temperature and 24-hour lighting for one week.

Shoot Culture in Thin-Layer System

The acclimatized Cavendish banana shoot cultures were cultivated in a thin-layer system with 25 mL medium. The medium used was half-strength MS, 20 g/L sucrose, and 0.5 ppm GA3. The initial weight (g) and height (cm) of the explants were measured. The culture was incubated on a shaker with a rotation speed of 60 rpm at room temperature and a 24-hour lighting period for 14 days. The experiment was conducted in triplicate.

Shoot Culture in Bubble Column Bioreactor

The acclimatized Cavendish banana shoot cultures were cultivated in a bubble column bioreactor with 200 mL medium. The medium used was the same as in the control system. The initial weight (g) and height (cm) of the explants were measured. Then, the air for aeration was transferred to the system using an air pump. A flow meter sets Aeration rates at 1 mL/s and 2 mL/s. This system was operated for 14 days at room temperature with a lighting period of 24 hours.

Plant Growth and Medium Analysis

The final weight (g) and height (cm) of the explants were measured after cultivating. The average growth rate of plant height and biomass were calculated as the difference between the initial and the final conditions, then divided by the cultivation time. Sucrose levels and conductivity in the cultivation medium were measured using a refractometer and conductivity meter, respectively.

Determination of Moisture Content and Extract Yield

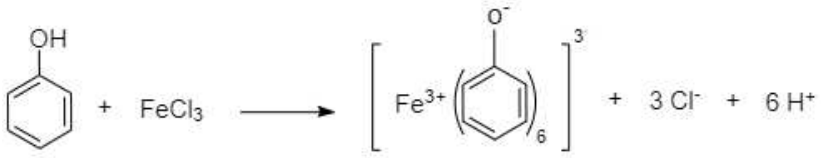
The leaves and stems of plantlets in the control and bioreactor after cultivating were then separated, and each was weighed as M_{fresh} (g). The plantlets were dried in an oven at 105°C for 15 hours [23]. Further, each part of the dry plantlet was weighed as M_{dry} (g). The moisture content was determined using (1) [23].

$$MC\ (\%) = \frac{M_{fresh}\ (g) - M_{dry}(g)}{M_{fresh}\ (g)} x\ 100\% \quad (1)$$

Dried plant parts were then crushed and extracted by the maceration method using 96% ethanol (1:10 (w/v) for 24 hours [24]. Hereafter, the solvent was evaporated using a rotary vacuum evaporator for 10-15 minutes to obtain the crude extract weighed as $M_{extract}$ (g) and the yield was determined using (2) [24].

$$\text{Extract yield}\ (\%) = \frac{M_{extract}\ (g)}{M_{dry}\ (g)} x\ 100\% \quad (2)$$

Then, the extract was dissolved in 96% ethanol and stored at -4°C for further analysis.



Phenolic compound Blue/green/black complex

Figure 1. The reaction of Phenolic Compound with $FeCl_3$

Determination of Phenolic Compound

The determination of phenolic compounds in banana leaf and stem extracts was carried out with a qualitative test using $FeCl_3$ [24, 25, 26]. A total of 1 mL of each extracted sample was taken and one drop of 5% $FeCl_3$ solution was added. Gallic acid solutions of 0,01 M and 0,5 mM were used as controls. The color change from brownish yellow to dark green indicates the presence of phenolic compounds in the extract, as shown in Figure 1.

Thin Layer Chromatography Analysis

Thin-layer chromatography (TLC) method was carried out based on Sonam et al. [25] with a modified TLC plate of silica gel 60 F254 (Merck, Germany) 7 cm × 3 cm. Gallic acid solutions were used as controls. First, the sample was applied to the silica plate in the lower limit and eluted using ethanol:water (6:3) as the mobile phase. Then, the plate was incubated until the solvent reached the upper limit of the plate. The Rf value is calculated using (3).

$$Rf = \frac{Stain\ mileage\ (cm)}{Solvent\ mileage\ (cm)} \quad (3)$$

Subsequently, 5% $FeCl_3$ solution was sprayed onto the plate surface to detect phenolic in the obtained stains. The existence of gray or blackish gray dots confirmed the presence of phenolic in the extracts [25].

Determination of Antioxidant Activity

An antioxidant test was carried out based on Ayoola et al. [5]. A total of 2 mL of banana leaf and stem extract at various concentrations (100, 50, and 25 µg/ml) and control were added to 3 mL of 0,1 mM DPPH solution. The solution mixture was vigorously stirred and incubated in the dark for 30 minutes at room temperature. The absorbance of the solution was measured using a spectrophotometer at 517 nm wavelength. The degree of color changes of the DPPH from purple to clear yellowish indicates the efficiency of the extract's free radical molecule reforming activity. The DPPH inhibition was calculated using (4).

$$\text{Inhibition } (\%) = \frac{A_{control} - A_{sampel}}{A_{control}} x\ 100\% \quad (4)$$

RESULTS AND DISCUSSION

Subculture and Acclimatisation

The cultivation of *Musa acuminata* shoot cultures in solid Murashige & Skoog (MS) medium with 0.5 and 1 ppm of *Gibberellic Acid* (GA3) hormones for four weeks aimed to prepare Cavendish banana explants that will be cultivated in the thin-layer system, as a role of the unaerated system, and bubble column bioreactor. After four weeks, banana explants on GA3 medium showed shoot elongation but did not differ much from each other, as depicted in Figure 2a. *Giberellic acid* (GA3) is a growth regulator which has an important role in

inducing shoot formation, promoting shoot elongation, and facilitating single node separation of plants [8, 27, 28].

Acclimatization of Cavendish banana shoots was carried out in a thin-layer system containing a half-strength MS liquid medium without hormones. It aimed to adapt the explants in a liquid medium before being cultivated into the treatment used, thus enabling an optimal response of banana plants after being cultured in the treatments [29]. After seven days of the process, the explants showed normal morphology and could be further used in a thin-layer system, which will be called TLS, and bubble column bioreactor as shown in Figure 2b.

Visual Analysis

Both control and bioreactor systems used half-strength MS liquid medium and 0.5 ppm of GA3 for 14-day cultivation, as shown in Figure 2c and Figure 2d. Visually, some explants in TLS showed hyperhydricity, where some of the leaves were light brown and died or abscised, as depicted in Figure 3b. Hyperhidricity is a physiological disorder in plant tissue cultured in vitro due to containing too much water [30]. In TLC, the liquid environment constantly soaking the explants will drastically reduce transpiration. As a result, the water absorbed from the medium is not transpired sufficiently and will accumulate in the intercellular tissue [30]. While in the bubble column bioreactor treatment, both flow rate variations resulted in a normal explant appearance after 14 days, where the leaves were green, and the stems were healthy, as shown in Figure 3c and Figure 3d.

Plant Growth Rate

The growth rate of Cavendish banana (*Musa acuminata*) in the bubble column bioreactor treatment was higher than the TLS. The highest increase in plant height was obtained in the flow rate variation of 1 mL/s, so as resulted in the largest average height growth rate of 0.04 ± 0.009 cm/day as listed in Table 1. The largest average biomass growth rate was also obtained in the flow rate variation of 1 mL/s, which was 0.22 ± 0.001 g/day, while the lowest was in the TLS.

a. b. c. d.

Figure 2. Shoot Culture of Cavendish Banana in a. Solid Medium, b. Acclimatization, c. TLS, d. Bubble Column Bioreactor

a. Before b. TLS c. 1 mL/s Rate After d. 2 mL/s Rate

Figure 3. Visual observation of Cavendish Culture in TLS and Bubble Column Bioreactor Treatment

Table 1. Average Growth Rate of Cavendish Banana in Bubble Column Bioreactor

Treatments	Δ Plant Height (cm)	Δ Biomass (g)	Average Growth Rate of Plant Height (cm/day)	Average Growth Rate of Biomass (g/day)
Flow rate of 1 mL/s	0,53 ± 0,125	3,02 ± 0,005	0,04 ± 0,009	0,22 ± 0,001
Flow rate of 2 mL/s	0,45 ± 0,250	2,94 ± 0,005	0,03 ± 0,025	0,21 ± 0,001

In the bubble column bioreactor, the lower flow rate will result in the minimum shear stress generated by bubble gases in the bioreactor. As a result, the medium's rate of gas transfer and nutrient transfer will increase [14]. Therefore, biomass production is higher than at higher flow rates [14]. Treatment in flow rate of 1 mL/s resulted in the highest increase in biomass growth rate compared to flow rate of 2 mL/s and TLS, which was 1.9 times greater than the initial average weight of 3.1 g in 14 days. This result is further supported by Esyanti et al. [29], who obtained a 1.8-fold increase in *Aquilaria malaccensis* shoot culture biomass using a small flow rate variation of 0.42 mL/s. The shoot growth rate depends on the medium's surface area in direct contact [31]. The bubble column bioreactor has a high probability of direct contact between the shoot surface area and the medium. Still, the shoots are not completely soaking in the liquid culture system, so it takes more energy to transfer nutrients to the meristematic tissue that is in direct contact with the medium [31]. In addition, the bubble column bioreactor also uses non-metallic agitation, which reduces shear stress and provides a continuous supply of oxygen. Nevertheless, the oxygen content in the medium from all treatments was not measured because it is implied that the increase of aeration rate will increase the rate of oxygen absorption in the medium [32][33].

Medium Analysis

TLS treatment resulted in the highest decrease of electrical conductivity (EC) than others, which was around 55%. EC is expressed in mS (milisiemens) and generally increases when the ion concentration in the medium is high [35]. According to Esyanti et al. [34], the decrease in the EC medium occurred because of nutrients absorption by the shoots for its growth. However, the results of this study are not by the statement, where TLS, which has the highest decrease in conductivity, produces the lowest growth rate of biomass. This allows for the influence of the remaining sucrose content in the final medium, where the EC of the medium is changed in parallel with the consumption of sucrose [31].

All treatments showed a decrease in the final medium pH after 14 days from the initial pH conditions, as listed in Table 2. The highest decrease was resulted in a flow rate variation of 1 mL/s, from 5.8 to 3.9. The decrease in pH indicates the consumption of nutrients in the form of ions by plants. Therefore, the pH conditions will affect the absorption of nutrients in the medium and regulate salt formation [8]. In media containing NO_3^- and NH_4^+ with an initial pH of 5-6, the uptake of NH_4^+ was preferred and caused the pH to drop during the initial growth of the culture [8].

All treatments generally showed a decrease in the final sucrose level, as shown in Table 2. The decrease in medium sucrose was exceedingly small in bubble column bioreactor treatment flow rate variations. At the same time, TLS resulted in the greatest decrease of sucrose level in the medium. Sucrose is a crucial factor for the growth of plant biomass on *in vitro* culture [29]. The decrease in sucrose at the end of the treatment indicated the use of carbon sources by plants. Sucrose acts as an energy source for growth, biosynthesis, and other metabolic processes [36, 37, 38]. The bubble column bioreactor was reported to have the most effective bioconversion compared to the TIS-RITA® bioreactor and thin-layer culture, with the lowest sucrose consumption, at 20%. In contrast, thin-layer consumed the highest sucrose, at 40% among the three [31].

Table 2. The Change of Conductivity, pH, and Sucrose in Medium

Treatments	Δ Conductivity (mS)	Δ pH	Δ Sucrose Level (g/L)
Flow Rate of 1 mL/s	0,05 ± 0,03	1,9 ± 0,10	0,33 ± 0,21
Flow Rate of 2 mL/s	0,23 ± 0,23	1,3 ± 0,00	0,12 ± 0,10

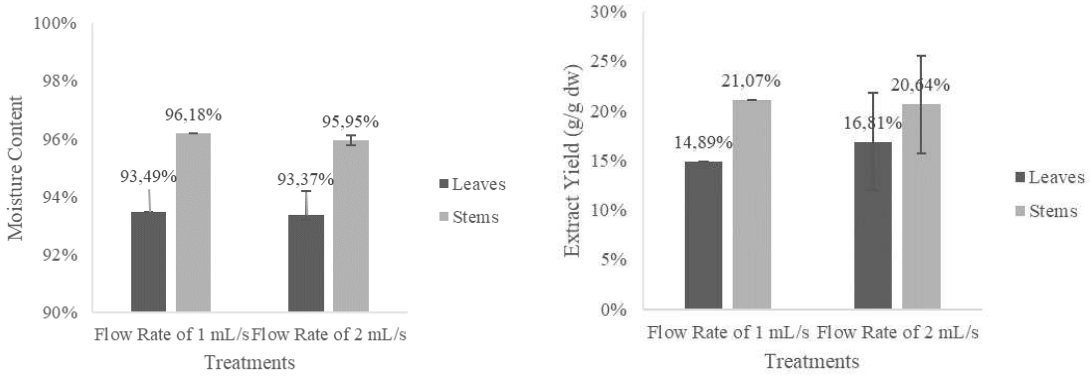


Figure 4. Moisture Content and Extract Yield of Cavendish Leaves and Stems

Moisture Content and Extract Yield

Figure 4 shows the results that all samples of Cavendish banana leaves and stems from each treatment had a moisture content of more than 90%. In general, banana leaves have a lower moisture content than banana stems. These results are in accordance with the research of Costa et al. [39] and Ramdhonee & Jeetah [23], who found that the moisture content in banana (*Musa spp.*) leaves and stems was 93.95% and 96.3%, respectively. Furthermore, the highest moisture content was observed in the sample of the TLS, while the lowest was in the bubble column treatment with a flow rate of 1 mL/s. This shows that in TLS, banana plants hold up more water than in the bioreactor treatment.

The percentage yield of crude ethanol extract in all treatments has various values between 14.89%-21.07%, as shown in Figure 4. Based on Ayoola et al. [5], the ethanol extract of banana leaves (*Musa spp.*) ranged from 6.15% to 42.04%. In general, stem samples at a flow rate of 1 mL/s produced the highest extract yields among all samples, while leaf samples' highest result was obtained at a flow rate of 2 mL/s. A high aeration rate is beneficial for accelerating oxygen transfer into the bioreactor, where the process increases the accumulation of secondary metabolites and cell growth [40, 41, 42].

Phenolic Test

Determination of phenolic compound with $FeCl_3$ was carried out as a first step to detect the presence of gallic acid compounds in a crude extract of Cavendish banana leaves and stems. The formation of a blue-green or blue-black color in the solution indicates a positive result for polyphenol compounds because phenol reduces Fe^{3+} to $Fe^{2+,}$ which causes a blue-black color (Iron (III) hesacyanoferrate) [43][44].

Figure 5 shows the crude ethanol extract of the banana leaf sample was greenish in color, while the stem extract had an orange to brownish color. However, Table 3 lists all extracts showed positive results for the phenolic test because they produced a green/blue color after being added with $FeCl_3$. However, the extract from TLS produced a very faded green color. These results indicated that banana leaves and stems in the bubble column bioreactor contain more phenolic compounds than TLS. All *Musa spp.* known to have a high amount of total phenolic content in the leaves, stems, flowers, and fruit, with the highest phenolic found in the leaves, which is about 64%, while the stem is only about 3% [44].

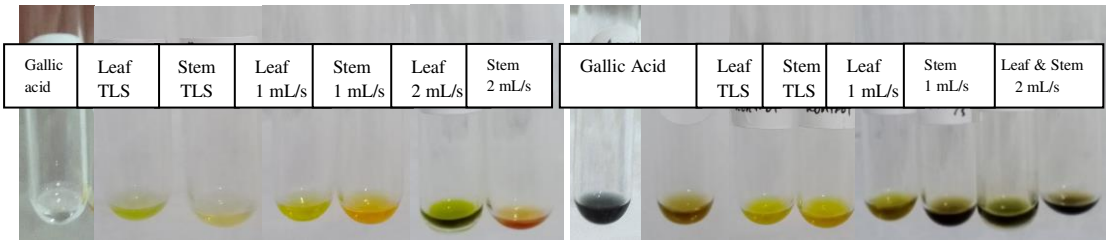


Figure 5. Result of Phenolic Test on Cavendish Leaf and Stem Extracts with $FeCl_3$

Table 3. Result of Phenolic Test

Treatments	Phenolic Test Result	
	Leaf	Stem
Flow rate of 1 mL/s	+	+
Flow rate of 2 mL/s	+	+

This factor makes the color changes in the leaf samples tend to be darker than the stems after the addition of $FeCl_3$. The difference in color between the gallic acid solutions, which is more blue-green, and the leaf samples may occur because of the presence of compounds other than polyphenols, such as flavonoids and tannins in the banana leaf samples [44]. A positive result of the tannin test with $FeCl_3$ is shown by a change in color to blackish green.

Thin Layer Chromatography

Thin layer chromatography (TLC) was performed to further determine the presence of gallic acid in each extract. TLC was carried out using ethanol:water (6:3) as the mobile phase and silica plate as the stationary phase [25]. Pure gallic acid solutions were used as comparisons. Thin-layer chromatography is commonly used to identify bioactive compounds, such as polyphenols, alkaloids, flavonoids, and tannins [25]. The stains produced by the extract samples and gallic acid solutions were both very faint brown in color.

Spraying of 5% $FeCl_3$ solution was carried out to confirm that the stains obtained in the TLC were phenolic compounds. Phenolic compounds in the ethanol extract will show a grey spot on the TLC plate after being sprayed with $FeCl_3$ [25]. The results showed that a pale grey spot was observed in the leaf extract from the 2 mL/s flow rate treatment as well as in the stem extracts from all flow rate variations with Rf values of 0.56 and 0.54, respectively after $FeCl_3$ spraying as depicted in Figure 6. However, the spots observed on the stems of the two flow rates have different Rf values with pure gallic acid. In polar solvents, compounds with higher Rf showed more polar extract properties than compounds with lower Rf values [25]. This allows the presence of other phenolic compounds than gallic acid contained in the extract. Meanwhile, no grey spots were observed in 0.1 mM gallic acid solution and extracts from the control treatment. This indicated that the leaf and stem extract of Cavendish banana in all treatments contained a very small amount of gallic acid so that the presence of gallic acid could not be detected by spraying $FeCl_3$.

Antioxidant Test

Antioxidants have anti-radical properties with scavenging activity against free radicals, which are useful for the treatment process in various diseases. Free radicals are the cause of many diseases, such as neurodegenerative diseases, cancer, and AIDS. The DPPH method is a sensitive method for determining the antioxidant activity of various plant extracts [22].

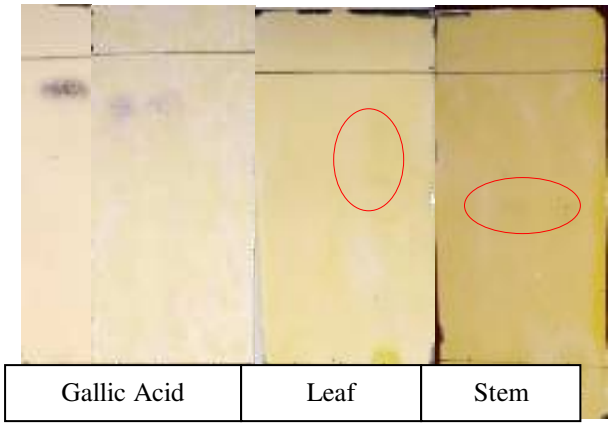


Figure 6. Result After Spraying $Fecl_3$ on TLC Plate

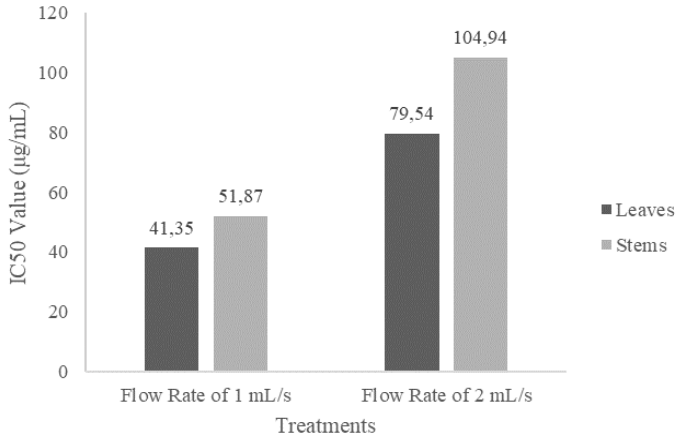


Figure 7. IC50 Value of Cavendish Leaf and Stem Extracts

Antioxidant activity is generally expressed in IC50 value, which is the concentration required to inhibit the formation of DPPH radicals by 50%. The IC50 value was calculated based on the equation obtained from the linear regression results on the percentage curve of DPPH inhibition to the sample concentration.

Figure 7 shows the highest antioxidant capacity was obtained in bioreactor treatment of 1 mL/s variation due to the smallest IC50 value obtained. The lowest antioxidant properties were observed in the extract of TLS. Antioxidant activity increased with increasing extract concentration and decreasing absorbance obtained. The lower the absorbance of the extract, the higher the capacity of the extract to donate electrons or hydrogen atoms to the DPPH compound. The IC50 results in this experiment are in accordance with Ayoola et al. [5], which obtained IC50 values between 9.57-323 g/mL in plant extracts of *Musa spp*. Meanwhile, the IC50 value of the gallic acid solution was negative, namely -413.56 g/mL. This can happen because of a very small absorbance of the pure gallic acid solution, and the solution concentrations used were outside the range of variations.

Several studies have shown a strong relationship between antioxidant activity and total phenolic compounds in plant extracts, referring to the important role of polyphenols as potential antioxidant biomolecules [45][46]. Antioxidant activity increases with increasing polyphenol content due to hydroxyl groups contained in phenolic compounds that play a role in the destruction of radical compounds, mainly because of their redox properties [46]. This statement indicates that banana leaves and stems at a flow rate of 1 mL/s have higher phenolic

compounds than those at a flow rate of 2 mL/s and TLC. Cultures in bubble column bioreactors produced metabolites like those of thin-layer cultures but in larger quantities due to increased respiratory energy caused by the input of oxygen into the bioreactor [34]. In addition, the higher antioxidant activity of the leaf extract than the stem in the bioreactor treatment could be due to polyphenols generally accumulating in the epidermal and subepidermal cells of the leaves and shoots [47].

CONCLUSION

Shoot culture of Cavendish banana (*Musa acuminata*) in bubble column bioreactor has a higher growth rate, phenolic content, and antioxidant activity than TLC. The average growth rate of biomass at a flow rate variation of 1 mL/s and 2 mL/s respectively was 0.22 ± 0.001 g/day and 0.21 ± 0.001 g/da. The phenolic content in banana leaf and stem extracts in the bubble column bioreactor was also higher than in the TLC. This result had implications for the presence of higher gallic acid compounds. However, gallic acid in the extracts could not be detected on thin layer chromatography because of the small amount of extract. The IC50 values for the banana leaf and stem extract were 41.35 and 51.87 g/mL for a flow rate of 1 mL/s; 79.54 and 104.94 g/mL for a flow rate of 2 mL/s.

ACKNOWLEDGMENT

The author would like to thank Banana Group for the research opportunity and LPIK ITB for partially funded the research.

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