OnLine Journal of Biological Sciences

Original Research Paper

Crude Extract of Blue-Green Pigment Derived from the Marine Bacterium *Pseudomonas Aeruginosa* P1.S9 has Antibacterial, Antioxidant, and Cytotoxic Activities

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Article history
Received: 20-09-2021
Revised: 12-02-2022
Accepted: 17-02-2022

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Abstract: Pigment derived from marine bacteria is one of the alternative sources of natural products characterized by beneficial functions for pharmaceutical purposes. This study aimed to evaluate the antimicrobial, antioxidant and cytotoxic activities of the marine bacterium *Pseudomonas aeruginosa* P1. S9 crude pigment extract and identify its chemical content. Crude pigment extract of this bacterium showed promising antibacterial activities against *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Escherichia coli*, and *Staphylococcus aureus* in a dose-dependent manner, as tested by the disc diffusion method. This extract also exhibited 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) radical scavenging activity with an IC_{50} value of 164.05 µg/mL. Furthermore, this extract also had toxicity in shrimp larvae *Artemia salina*, with an LC_{50} value of 122.11 µg/mL, as evaluated by Brine Shrimp Lethality Test (BSLT). These results were supported by a cytotoxicity test against MCF-12A (human mammary epithelial cells) and MCF-7 (human breast adenocarcinoma cells) with an IC_{50} value of 265.47 and 171.98 µg/mL, respectively. GC-MS analysis identified that trans-2-decenoic acid was found as the most dominant compound in this extract.

Keywords: Antibacterial, Anti-cancer, Antioxidant, Bacterial-Pigment, Marine Bacteria

Introduction

The marine environment is known to have a unique microbial community that is significantly different from the terrestrial environment. Microbes living in this environment must be able to survive and grow in the condition of high salinity, low nutrition, and high pressure. These conditions may influence their ability to produce bioactive compounds with various biological properties. Therefore, the bioactive compounds derived from marine bacteria are becoming a research hot spot due to their useful properties, particularly for pharmacological use (Hamid and Rosmadi, 2020; Bose *et al*., 2015). In our previous studies, marine bacteria associated with sponges have been reported to have numerous pharmacological activities. For example, *Bacillus* sp. isolated from sponge *Japsis* sp. showed antioxidant and anti-aging activities (Prastya *et al*., 2018). Several bacteria isolated from sponges *Hyrtios* sp., *Verongula* sp., and *Smenospongia* sp, exhibited anti-*Vibrio* activity (Wahyudi *et al*., 2018). In addition, the sponge-associated bacteria *Bacillus subtilis* also was able to produce anti-cancer compounds (Priyanto *et al*., 2017). Among marine bacteria species, marine *Pseudomonas* is considered one of the biotechnological potential groups that could produce versatile compounds, including enzymes, bioactive substances, and biosurfactants (Isnansetyo and Kamei, 2009).

Pseudomonas aeruginosa draws attention due to its pigmentation. This bacterium belonged to a Gram-negative group that was able to produce various soluble pigments such as pyocyanin (blue-green), pyoveridin (yellow-green), pyorubin (red), and pyomelanin (brown). However, the most common pigment produced by this bacterium is pyocyanin, a water-soluble phenazine pigment group that is secreted extracellularly as a secondary metabolite (Gonçalves and Vasconcelos, 2021). In the medicinal field, this pigment has been reported to have various biological activities, including inducing dose-dependent apoptosis of human pancreatic cancer cell line (Panc-1), broad-spectrum antimicrobial (Hamad *et al*., 2020), antioxidant, antimalarial, immunosuppressive, antiparasitic and antibiofilm

activities (Jayaseelan *et al*. 2014; DeBritto *et al*., 2020; Ozdal 2019; Wu *et al*., 2014).

In our previous study, *P. aeruginosa* P1.S9, a blue-green pigmented bacterium has been successfully isolated from a marine sponge and known to have potential anti-vibriosis in shrimps (Rini *et al*., 2017). However, the potencies of the pigment extracted from this bacterium in the pharmaceutical field were yet to be investigated. Here, our present study aimed to evaluate biological properties, including antimicrobial, antioxidant, and cytotoxic activities, and identify the chemical composition of the crude pigment extract derived from *P. aeruginosa* P1.S9.

Materials and Methods

Materials

Pseudomonas aeruginosa P1.S9 used in this study has been isolated from a marine sponge collected from Kepulauan Seribu, Indonesia (Rini *et al*., 2017). Five microbes used for the antimicrobial test were *Bacillus subtilis* ATCC 19659, *Escherichia coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 15442, *Staphylococcus aureus* ATCC 6538, and *Candida albicans* ATCC 10231 obtained from IPB Culture Collection (IPBCC), IPB University (Bogor, Indonesia). The human breast adenocarcinoma cells (MCF-7 ATCC CRL-1072) and human mammary epithelial cells (MCF-12A ATCC HTB-22) were obtained from Primate Research Center, IPB University (Bogor, Indonesia).

Methods

Extraction of Bacterial Pigment

The bacterial pigment extraction was carried out based on the previous method described by Karwati *et al.* (2015) with modifications. The *P. aeruginosa* P1.S9 was cultured in 1 L of Seawater Complete (SWC) medium (5 g of peptone, 3 mL of glycerol, 1 g of yeast extract, 750 mL of seawater, 250 mL of distilled water) and incubated on a rotary shaker at 120 rpm at room temperature (±28°C) for seven days. The extraction of bacterial pigment was carried out by adding ethyl acetate into the culture with a 1:1 (v/v) ratio. The mixture was then shaken continuously for 20 min. The ethyl acetate phase was evaporated at 50°C and the crude pigment extract was stored at 4°C.

Antimicrobial Assay

The antibacterial assay was performed based on the disc-diffusion method. The crude pigment extract was tested against *B. subtilis* ATCC 19659, *E. coli* ATCC 8739, *P. aeruginosa* ATCC 15442, *S. aureus* ATCC 6538, and *C. Albicans* ATCC 10231. The tested bacteria were inoculated into Mueller-Hinton Agar (MHA) while *C. albicans* ATCC 10231 was inoculated into Potato Dextrose Agar (PDA). A 6 mm paper disc containing several extract concentrations (250, 500, 750, and 1000 µg/mL) diluted with 10% Dimethyl Sulfoxide (DMSO), was placed on the agar surface. Ampicillin (100 µg/mL), nystatin (20000 µg/mL), and 10% DMSO were used as a positive control in bacteria strains inoculated plates, positive control in *C. Albicans* inoculated plates, and negative control, respectively. The inoculated plates were then incubated at ±37°C for Gram-positive and Gram-negative tested strains and ±28°C for *C. Albicans* strain for 24 h, then the formed clear zone was observed and the diameter was measured.

Antioxidant Assay

The DPPH assay used in this study refers to the method described by Kandi and Charles (2019) with modifications. A total of 500 µL of the extract at various concentrations (0, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 µg/mL) was mixed with 500 µL of 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) reagent (0.125 µm diluted in methanol). The mixture was incubated for 30 min in a dark condition. The absorbance of the sample was measured by a spectrophotometer (Thermo Spectronic-Genesis 20, Thermo Fisher Scientific, USA) at 514 nm. Ascorbic acid was used as a positive control. The percentage of inhibition was calculated using the following formula:

$$Inhibition(\%) = \left(1 - \frac{Sample\ absorbance - control\ absorbance}{Blanko\ absorbance - control\ absorbance}\right) \times 100$$

The IC_{50} value of the extract was determined by a linear regression model.

Toxicity Assay

The toxicity assay was performed by Brine Shrimp Lethality Test (Meyer *et al*., 1982). Twenty *Artemia salina* larvae were put in each vial tube containing 4 mL seawater and crude pigment extracts at various concentrations (0, 10, 100, 250, 500, 750, 1000 µg/mL). The vials were then incubated for 24 h at room temperature under a light. The dead and surviving larvae were counted and the mortality percentage was calculated using the following formula:

$$\%\,mortailty = \left(\frac{\sum sample\ larvae\ mortality - \sum control\ larvae\ mortality}{\sum total\ larvae}\right) \times 100$$

The median lethality concentration (LC_{50}) was statistically analyzed using probit analysis and regression linear model.

MTT Cytotoxicity Assay

The cytotoxic property of the crude pigment extract from *P. aeruginosa* P1.S9 was determined using the Microculture Tetrazolium Test (MTT). One hundred microliter culture containing MCF-7 and MCF-12A cell lines (5 x 10^3 cell/well) were cultured in Rosewell Park Memorial Institute medium 1640 (RPMI 1640), supplemented with 5% Fetal Bovine Serum (FBS), 20% penicillin (100 U/mL) and streptomycin 100 µg/mL and incubated for 24 h. A total of 100 µL of extract in various concentrations (25, 50, 100, 200, 400, and 800 µg/mL) was added to the plate and incubated for 24 h. Ten microliters of 3-[4,5-dimetiltiazol-2-il]2,5-difeniltetrazolium bromide (MTT) were added to the cell mixture and incubated for 4 h. The culture was then diluted in 100 µL of 96% ethanol. The absorbance of each plate was measured by an ELISA reader (Biorad iMark, USA) at 595 nm. The inhibition percentage of cells growth was calculated using the following formula:

$$Inhibition(\%) = \frac{Absorbance\,of\ control - Absorbance\,of\ sample}{Absorbance\,of\ control} x100$$

The inhibitory concentration 50 (IC_{50}) value of the extract was determined by a linear regression model. The IC_{50} value is the extract concentration that is effective in inhibiting 50% of cell line viability.

Identification of Bacterial Pigment Components

The chemical profile of the crude pigment extract was performed by GC-MS analysis Agilent 5977B GC/MSD (Agilent Technologies, USA). A total of 0.6 µL of the extract dissolved in ethyl acetate was injected into an HP-5 MS column (30 x 250 x 0.25 µm). The oven temperature was initially set at 80°C and gradually increased at the rate of 15°C/min until 300°C and held for 20 min. Helium gas was used as a carrier at a 1 mL/min flow rate. The injection temperature was kept at 300°C and the aux temperature was maintained at 300°C. The GC-MS Pyrolysis Program (WILLEY9THN 08. L) was used to analyze the results.

Results

Crude Pigment Extract of P. aeruginosa P1.S9

Pseudomonas aeruginosa P1.S9 showed blue-green colonies on SWC agar on the second day of incubation at room temperature. The crude pigment extracts showed dark blue-green color (Fig. 1). The crude pigment yield of the bacteria was 0.017% (w/v).

Antimicrobial Activities

The antimicrobial assay exhibited crude pigment extract of *P. aeruginosa* P1.S9 had antibacterial activity to inhibit the growth of four tested bacteria, which was indicated by inhibition zone formation (Table 1; Figure 2) and had no activity against *C. Albicans*. A larger inhibition zone was found in the higher crude pigment extract concentration used. This extract had active antibacterial action against both Gram-negative and Gram-positive bacteria. The maximum antibacterial activity of all targeted bacteria was found in the extract concentration of 1000 µg/mL.

Antioxidant Property

Pseudomonas aeruginosa P1.S9-derived crude pigment extract possessed scavenging activity against 1,1-Diphenyl-2-Picrylhydrazil (DPPH) with an IC_{50} value of 164.05±1.74 µg/mL (Table 2).

Toxicity of Pigment Extract

The BSLT (Brine Shrimp Lethality Test) was used to determine the compound's ability to be toxic to living cells. The standard LC_{50} calculation is used in the study to determine how toxic the compound is in crude pigment extract. Our result showed that the crude pigment extract of *P. aeruginosa* P1.S9 had an LC_{50} value of 122.11±24.19 µg/mL (Table 2).

Cytotoxic Activity

Cytotoxicity assay showed that the crude pigment extract of *P. aeruginosa* P1.S9 inhibited 50% proliferation of MCF-12A and MCF-7 cells in the concentration of 265.45 µg/mL and 171.98 µg/mL, respectively (Table 3).

Chemical Components of Pigment Extract

According to GC-MS analysis, the crude pigment extract of *P. aeruginosa* P1. S9 was dominated by the fatty acid group with trans-2-Decenoic acid as the most dominant compound (Table 4; Fig. 3).

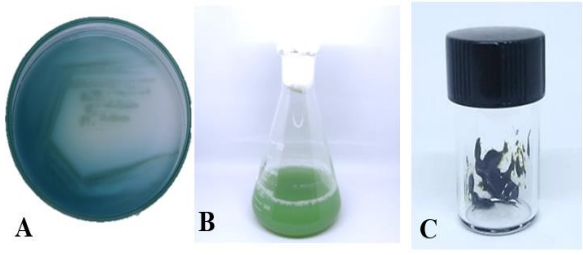

Fig. 1: *P. aeruginosa* P1. S9 colonies in Seawater Complete (SWC) agar at the second-day incubation (A); *P. aeruginosa* P1.S9 culture in SWC broth after 7 days incubation (B); and its crude pigment extract (C)

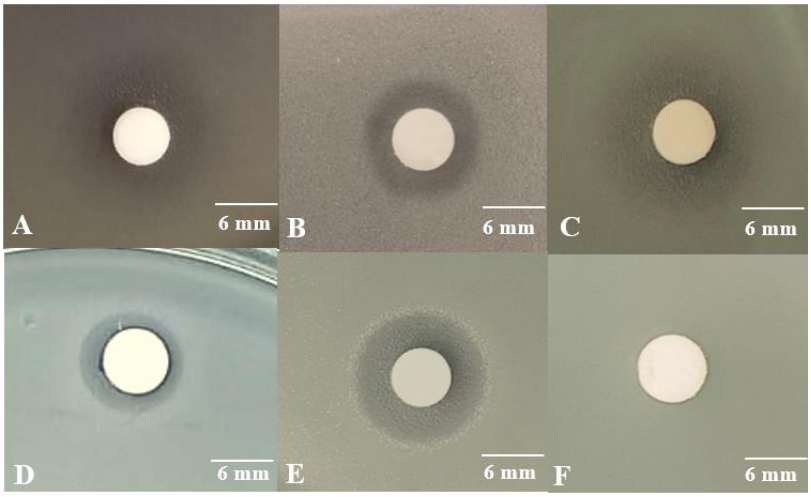

Fig. 2: Antibacterial activity of *P. aeruginosa* P1.S9-derived crude pigment extract (1000 µg/mL) against *P. aeruginosa* (A); *B. subtilis* (B); *S. aureus* (C); *E. coli* (D), compared to the ampicillin 100 µg/mL against *B. subtilis* (E) and DMSO 10% (F*)* against *B. subtilis* after 24 h incubation at ±37°C

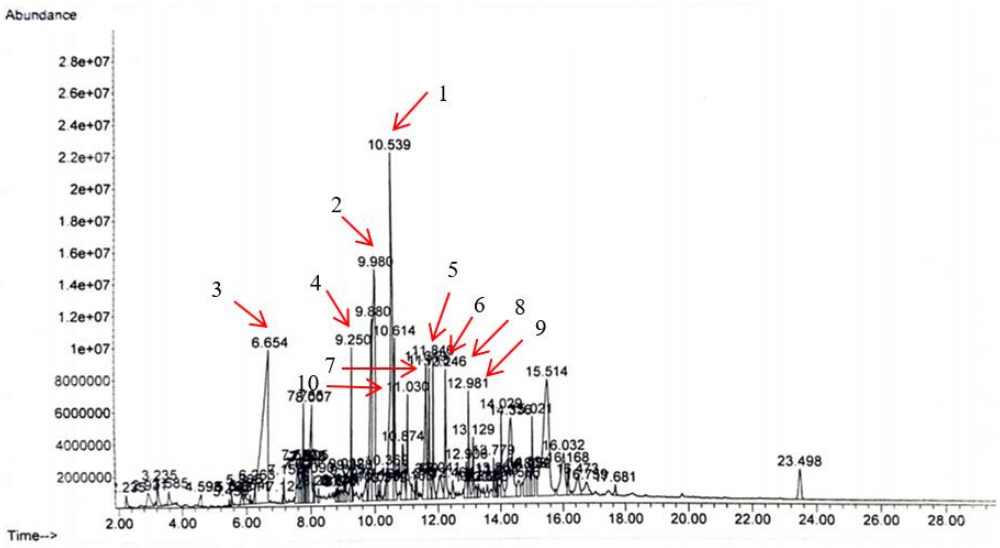

Fig. 3: GC-MS chromatogram profile of crude pigment extract from *P. aeruginosa* P1.S9 showing the ten most dominant compounds indicated by red arrows

Table 1: Antimicrobial activity of crude pigment extract derived from *P. aeruginosa* P1.S9

		Inhibition zone (mm) ± SD*				
Sample	Concentration	*P. aeruginosa* ATCC 15442	*B. subtilis* ATCC 19659	*E. coli* ATCC 8739	*S. aureus* ATCC 6538	*C. albicans* ATCC 10231
Crude pigment extract	0 (µg/mL)	0	0	0	0	0
	250 (µg/mL)	2.67±3.77	4.67±3.30	0	8.33±1.25	0
	500 (µg/mL)	8.00±0	9.67±1.25	8.00±1.63	10.00±0.00	0
	750 (µg/mL)	11.33±1.89	12.00±1.63	8.33±0.47	13.33±1.25	0
	1000 (µg/mL)	10.67±0.94	11.67±1.25	10.33±0.47	15.67±0.47	0
Ampicillin	100 (µg/mL)	14.67±0.47	24.67±0.47	15.00±0	30.67±0.94	-
Nystatin	20000 (µg/mL)	-	-	-	-	25±0
DMSO	10 % (v/v)	0±0	0±0	0±0	0±0	0±0

Table 2: Antioxidant property and toxicity of *P. aeruginosa* P1.S9 crude pigment extract

No.	Isolate name	Antioxidant property IC_{50} value (µg/mL) ± SD	Toxicity LC_{50} value (µg/mL) ± SD
1.	*P. aeruginosa* P1.S9	164.05±1.74	122.11±24.19

Table 3: Cytotoxic property of crude pigment extracted from *P. aeruginosa* P1.S9

No.	Cell lines	IC_{50} (µg/mL)* 1	2	3	Average of IC_{50} (µg/mL) ± SD
1.	MCF-12A	259.15	282.72	254.54	265.47 ± 15.12
2.	MCF-7	144.06	194.61	177.28	171.98 ± 25.69

*IC_{50} value obtained from each replication

Table 4: Chemical profile of *P. aeruginosa* P1.S9 crude pigment extract

No.	Compounds	Peak area (%)	Retention times (min)	Biological activities	References
1	Trans-2-Decenoic acid	11.66	6.654	Antioxidant, anti-inflammatory	Ali and Hendawy (2019)
2	Hexadecanoic acid (CAS)	9.930	10.539	Antimicrobial, antifungal	Prasath *et al*., (2020); Umaiyambigai *et al.* (2017)
3	Caffeine 1H-Purine-2,6-dione, 3,7-dihydro-1,3,7-trimethyl-	8.250	9.980	Antioxidant	Vieira *et al*., (2020)
4	Octadecenoic acid ester	3.410	11.619	Antiviral, antibacterial, antioxidant	Sudharsan *et al*., (2010)
5	Oleic acid	2.120	10.030	Antibacterial, antioxidant	Karimi *et al*., (2015)
6	18-Nonadecanioc acid	2.070	12.246	Antioxidant	Fadeyi *et al*., (2015)
7	Octadecenoic acid (CAS)	1.940	11.713	Antibacterial, antifungal	Pradheesh *et al*., (2017)
8	1-Nonadecane	1.520	11.846	Antibacterial, antifungal	Naragani *et al*., (2016)
9	1-Octadecane (CAS)	1.440	9.250	Antimicrobial, antifungal	Abubacker (2017)
10	Cyclotetracosane	1.110	12.981	Antibacterial	Gumgumjee and Hajar (2012)

Discussion

In our present study, *P. aeruginosa* P1.S9 isolated from marine sponge produced blue-green pigment in SWC agar after 48 h incubation. Crude extract of this pigment showed broad spectra of antibacterial activity against both Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilis* ATCC 19659, *Escherichia coli* ATCC 8739, and *Staphylococcus aureus* ATCC 6538. The antibacterial activity of this extract increased by increasing extract concentration from 250 to 1000 µg/mL. This extract showed more inhibitory activity against Gram-positive bacteria with maximum antibacterial activity found against *S. aureus* ATCC 6538 in the concentration of 1000 µg/mL, as indicated by the largest clear zone diameter. The difference in susceptibility could be influenced by the distinctive cell wall structure between Gram-positive and Gram-negative bacteria. The higher component of lipopolysaccharide might give more protection to Gram-negative bacteria. Consequently, this bacteria group is less sensitive to antibacterial compounds (Breijyeh *et al*., 2020). In contrast, Gram-positive bacteria lack this important layer, consequently, this bacteria group is more sensitive to an antimicrobial agent (Epand *et al*., 2016). However, this extract was no effect on the growth of *C. Albicans*. This effect might be influenced by its resistance to crude pigment extract used in this study and is also likely caused by the significant differences in cell wall components between yeast and bacteria. Supporting the results of this study, Darwesh *et al.* (2019) reported that blue-green pigment synthesized by marine *P. aeruginosa* also showed antimicrobial activity against several pathogenic microorganisms, such as *Aeromonas hydrophila*, *Escherichia coli*, *Vibrio cholerae*, *Staphylococcus aureus* MRSA, *Staphylococcus aureus*, *Pseudomonas* sp. *Listeria monocytogenes*, *Staphylococcus lentus*, *Candida albicans*, *Candida Tropica* and *Fusarium oxysporum.*

To analyze other biological properties of pigment derived from *P. aeruginosa* P1.S9, we assessed antioxidant activity against DPPH radicals *in vitro*. As a result, the IC_{50} value of this extract was 164.05 µg/mL, higher than ascorbic acid with an IC_{50} value of 7.39 µg/mL. In contrast, a study done by Saleem *et al.* (2021) revealed that purified pigment from *Pseudomonas* species had a better scavenging activity than ascorbic acid and Trolox. The contrast result can be explained that our pigment extract had not been purified yet. Therefore, further study to purify this extract is needed to be done.

Cytotoxicity of the crude pigment extract was pre-screened by the Brine Shrimp Lethality Test (BSLT). Based on the result, the extract is categorized as a toxic compound with an LC_{50} value of 122.11 µg/mL. Substances with low LC_{50} values belonging to a biologically active compound could be a candidate for antibiotics and anticancer. Supporting this result, a previous study revealed

the toxicity of purified pigment from *P. aeruginosa* had an LC_{50} value of 292.11 µg/mL (Hamad *et al*., 2020).

A positive correlation between toxicity properties and anticancer activity was shown that *P. aeruginosa* P1.S9 also had cytotoxic properties against MCF-12A and MCF-7 cell lines. The extract inhibited 50% cell proliferation at a concentration of 265.47 and 171.98 µg/mL for MCF 12A and MCF-7, respectively. This value indicates that this extract is relatively more active to inhibit cancer cell line growth than normal cells. Pigment extract derived from marine bacteria also exhibited cytotoxicity against colon cancer cell line (HCT_{15}) with an IC_{50} value of 255.58±43.51 mg ml^{-1} (Aroumougame, 2021). Therefore, the marine bacterial pigment may play an important role in inhibiting cancer cell proliferation.

In this study, we identified the chemical profile of the crude pigment extract from *P. aeruginosa* P1. S9 by GC-MS analysis. Our results showed fatty acid group compounds were found as major compounds in this extract. The two most abundant compounds, trans-2-decenoic acid and hexadanoic acid (CAS) may play a crucial role in antioxidant, anticancer, and antibacterial properties, which are supported by previous investigations (Ali and Hendawy, 2019; Prasath *et al*., 2020; Umaiyambigai *et al*., 2017; Bharath *et al*., 2021). Medium and long-chain fatty acids also have been well studied to have potential antimicrobial activities (Huang *et al*., 2011). Considering the pharmacological properties of crude pigment extract from *P. aeruginosa* P1.S9, this isolate is a potential source of biologically active compounds and needs to be further studied, particularly its potential for biomedical applications.

Conclusion

Crude pigment extract derived from *P. aeruginosa* P1.S9 has been characterized to have wide biological activities, including antibacterial, antioxidant, and cytotoxic activities. This extract was dominated by fatty acid compounds, particularly trans-2-decenoic acid which is well investigated as a biologically active compound.

Acknowledgment

We gratefully thank the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia for all the support.

Funding Information

This study was financially supported by the Basic Research ("*Penelitian Dasar*") 2021/2022 from the Ministry of Education, Culture, Research and Technology of the Republic of Indonesia to ATW.

Author's Contributions

Aris Tri Wahyudi: Has led the project, designed the research activities, and was involved in paper writing.

Rahmah Nursari: Has been involved in the experimental works and data analysis.

Widya Esti Purwaningtyas: Has been involved in data analysis and paper writing.

Uci Cahlia: Has contributed to the paper writing

Jepri Agung Priyanto: Has contributed to the interpretation of data dan paper writing.

Ethics

This article is authentic to the authors' works. The corresponding author ensures that all of the other authors have read and recognized the manuscript.

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