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Research article

Functional Compounds of Chili (*Capsicum annuum* L.) Fruit in Various Maturity Stages

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Abstract

Chili is a horticultural commodity with high economic value. Chili consumption starts from immature fruit to mature fruit for spicy food flavor. Physiologically, differences in chili maturity stages affect the secondary metabolites and potentially have a positive correlation effect on human health. The aim of this study was to identify the functional biochemicals (polyphenol, flavonoid, antioxidant activity, α-glucosidase inhibitory) at chili's three maturity stages. The experimental design was a factorial randomized complete block design with two factors: genotype and maturity stage. The three maturity stages were immature fruit, intermediate, and mature fruit. Eight chili genotypes were used; all were of *Capsicum annuum* L. species. Functional biochemical measurements consisted of total phenolics (TPC), total flavonoids (TFC), DPPH antioxidant activity (DPPH), FRAP antioxidant activity (FRAP), and α-glucosidase inhibitory (AGI) activity. The results showed that immature chili fruit had high DPPH and α-glucosidase inhibitory antioxidant activities. However, phenolic content, flavonoids, and FRAP antioxidant activity increased from immature to mature fruit. Immature ornamental chilies with purple fruit color were identified as having 75% α-glucosidase inhibitory activities. This research information can be used as a reference for further chili fruit biomedical development. In addition, chili breeders can use this information to determine preferences in developing new varieties.

Keywords: antioxidant activities; α-glucosidase inhibitory; maturity stages; ornamental chili; polyphenol content

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1. Introduction

Chili consumers are spread worldwide because of the spicy sensation produced by the fruit of the chili plant. Generations from Silent Generation to Alpha Generation have been identified as consuming chili as a flavor enhancer in food (Toledano et al., 2023). The main compound controlling spiciness is capsaicin found in chili seeds, flesh, and placenta (Tanaka et al., 2021). The latest facts reveal that capsaicin is a compound that causes inflammation and thus causing a spicy sensation. In addition to capsaicin, potential compounds beneficial to human health have been identified in chili, such as polyphenols, flavonoids, carotenoids, tocopherols, ascorbic acid, and antioxidant activity (Villa-Rivera & Ochoa-Alejo, 2020; Azlan et al, 2022; Alonso-Villegas et al., 2023). Recent research indicates that chili contains functional compound, namely α-glucosidase inhibitory (Sahid et al., 2023). These compounds are enzymes that reduce the absorption of sugar into the bloodstream (Zhong et al, 2024). This mechanism works by blocking the breakdown of carbohydrates, causing glucose to be excreted directly in the urine or feces.

Genetic and environmental factors determine the chili fruit functional compounds (Uarrota et al., 2020; Arce-Rodríguez et al., 2021). Genetic factors are distinguished by varieties and species (Tripodi et al., 2020). Functional biochemical information on chili fruit was studied widely during post-COVID-19 which impacted global health. Metabolomic research on chili as a functional food has shown promising potential to help address the growing number of diabetic patients due to diet. Chili is a high-economic horticultural plant. Chili consumption is divided into three stages based on the fruit maturity: immature, intermediate, and mature (Vázquez-Espinosa et al, 2020). Fruit maturity difference levels cause differences in its secondary metabolites, supported by several studies on other commodities (Alenazi et al., 2020; Yusuf et al., 2021; Zhang et al., 2022; Yue et al., 2024)

The chili maturity stage affects the physiological changes in producing secondary metabolites. Plants produce secondary metabolites as reserve compounds to maintain the life cycle (Salam et al., 2023). There are two phases in plants: vegetative and generative. Vegetative focuses on producing primary metabolites as a support to the generative. Entering the generative, plants produce more complex metabolites. For example, onions produce allicin compounds (Borlinghaus et al., 2021), tomatoes produce lycopene (da Costa et al., 2023), carrots produce vitamin A and beta-carotene (Anjani et al., 2022), and chili produces capsaicin (Batiha et al., 2020). In addition to the main compounds as primary metabolites, plants produce secondary metabolites to adapt to environmental stress due to global warming effects. In stressed conditions, plants produce more secondary metabolites, positively affecting human health.

A number of plant secondary metabolites studied have many benefits for human health. As noted, chili fruit at three maturity stages (immature, intermediate, and mature fruit) can be consumed. The consumption of immature chili fruit reduces spiciness, while mature fruit produces perfect spiciness. Information on functional biochemicals at the chili fruit maturity stage is still limited. Thus, understanding the functional biochemicals present in the chili fruit at maturity stage is crucial to providing the best fruit quality for consumers. This study aimed to evaluate and determine functional biochemicals in chili at three maturity stages. The results should help to determine the optimal maturity stage for chili fruit harvesting with better quality.

2. Materials and Methods

2.1 Study area and genetic material

This research area was specific to determining functional metabolomic analysis, including total phenolic content, flavonoids, antioxidant activities, and glucosidase inhibitory activity at the chili fruit maturity stages. The research activity began with planting eight chili genotypes in the IPB Alam Sinarsari D80 greenhouse. The experimental design was a factorial randomized complete block design with two factors (genotypes and chili fruit maturity stages). The genotypes used in this study are shown in Table 1. The chili fruit maturity stages used for functional metabolomic analysis were three stages (immature, intermediate, and mature stages). The selection of these three stages of chili fruit maturity was based on previous research reports that showed capsiate changes in chilies (Vázquez-Espinosa et al., 2020). The eight genotypes used were based on results from previous biochemical and phylogenetic analysis

Table 1. The chili genotypes for functional metabolomic analysis

No	Chili Code Genotypes	Species
1	IPB005	*Capsicum annuum*
2	IPB374	*Capsicum annuum*
3	IPB367	*Capsicum annuum*
4	IPB435	*Capsicum annuum*
5	IPB439	*Capsicum annuum*
6	IPB074	*Capsicum annuum*
7	IPB141	*Capsicum annuum*
8	IPB363	*Capsicum annuum*

Functional metabolomic analysis was repeated three times based on the experimental design in the greenhouse. Each replication used six sample plants to harvest chili fruit per genotype (2 plants were used for each maturity stage). A total of ten chili fruits were utilized for functional metabolomic measurements harvested from the third to fifth nodes on the sample plants based on the maturity stages of the chili. Functional metabolomic measurements were conducted at the Biopharmaceutical Laboratory (TROP-BRC), IPB University. The chili planting procedure refers to previous research, and the fresh fruit harvested was immediately analyzed in the laboratory (Syukur et al., 2024).

2.2 Functional compounds procedure

2.2.1 Sample preparation

The first sample preparation step was drying fresh chili fruits in an oven at 45ºC until the fruit water content reaches 10.0-12.5%. Thus, the functional metabolites did not decrease (Khuriyati et al., 2022). The next step was to grind the dried chili-fruits into chili powder with 15-20 mesh. Then, 10 g of chili powder was put into a glass beaker, dissolved in 110 mL of 70% ethanol and shaken using an orbital shaker for 48 h. The homogeneous solution was then filtered using a Whatman No. 42 filter, and the extract volume was calibrated to 100 mL to be used as a 1000 ppm stock extract solution. The extract stock solution was stored in a refrigerator at 5ºC.

2.2.2 Functional compounds measurement

The stock extract solution was diluted at various concentrations (10, 100, 250, 500, 750, 1000 ppm) to calibrate the sample solution used for functional metabolomic measurements (total phenolic content, total flavonoid content, antioxidant activity using the DPPH method, antioxidant activity using the FRAP method, and α-glucosidase inhibitory). After obtaining the right concentration, the standard curve calibration was carried out using standard solutions for each variable.

The standard used was: gallic acid for total phenolic content (TPC), quercetin for total flavonoid content (TFC), Trolox for antioxidant activities by two methods (DPPH and FRAP), and acarbose for α-glucosidase inhibitory (AGI) activity. Metabolomic measurements were based on previous research using microplate reader spectrophotometry (Khuriyati et al., 2022). The wavelengths of the functional compounds measurement were varied: total phenolic content used 750 nm, total flavonoid content used 415 nm, DPPH antioxidant activity used 517 nm, FRAP antioxidant activity used 595 nm, and α-glucosidase inhibitory activity was measured at 410 nm.

2.3 Data analysis

Microsoft Excel Office Home Student 2019 was used to convert the absorbance data obtained from the spectrophotometer. Absorbance conversion was carried out using the results of standard curve calculations for each compound. After obtaining the quantitative data, ANOVA and Post-Hoc tests were carried out using SAS On Demand for Academics as the leading software. Mini Tab and R Studio software were also used in this study to increase data validity.

3. Results and Discussion

The analysis of variance showed that genotype, fruit maturity stage, and the interaction between the two had a significant effect at the 1% level on all observed functional biochemicals. Replication did not have a significant effect on DPPH and FRAP antioxidant activities. However, it significantly affected total phenolic content, total flavonoid content, and α-glucosidase inhibitor activity. The results of this ANOVA confirmed that statistically, the Post-Hoc test could be performed on sources (genotype, fruit maturity stage, and their interaction). The coefficient of variation identified ranged from 1.54% to 7.07% in the ANOVA test (Table 2).

Information on functional biochemicals of maturity chili fruit is still very limited. The data analysis in this study used ANOVA as the initial step, followed by a PostHoc test. The ANOVA results showed a coefficient of variation below 10%, indicating that the statistical distortion in this study was very small because the chili used were the same species. The diversity obtained is more significant if there are differences in species but still in the same genus (Farwah et al., 2020). Functional biochemicals carried out at different levels of chili maturity can provide information on the optimum level of fruit maturity for consumption with high functional biochemicals.

Table 2. Anova for chili functional biochemicals

Sources	d.f.	TPC	TFC	DPPH	FRAP	AGI
Repetition	2	20,46*	0,09*	0,0001^{ns}	1,82^{ns}	76,14*
Genotypes (G)	7	195,06**	0,24**	0,01**	51,54**	3448,96**
Mature Stages (M)	2	932,17**	22,97**	0,60**	2942,74**	1662,92**
G x M	14	19,86**	0,56**	0,021**	54,23**	48,99**
Error	46	3,938	0,026	0,001	1,225	9,854
Coefficient of Variance (%)		7,07	6,44	1,54	3,14	6,98

[1] d.f. = degree of freedom; [2] TPC = total phenolic content; [3] TFC = total flavonoid content; [4] DPPH = antioxidant use DPPH method; [5] FRAP = antioxidant use FRAP method; [6] AGI = inhibitory α-glucosidase activity, ** = significance at α: 1%; * = significance at α: 5%; ns = non-significance.

Information on total phenolic content in the three stages of chili fruit maturity is shown in Table 3. The order of genotypes for TPC from highest to lowest was IPB 363 (34.06 mg GAE g^{-1} extract), IPB 141 (33.90 mg GAE g^{-1} extract), IPB 439 (29.59 mg GAE g^{-1} extract), IPB 367 (29.52 mg GAE g^{-1} extract), IPB 374 (27.89 mg GAE g^{-1} extract), IPB 435 (24.69 mg GAE g^{-1} extract), IPB 074 (23.31 mg GAE g^{-1} extract), and IPB 005 (21.48 mg GAE g^{-1} extract).

The highest total phenolic content was observed in the mature fruit. The result was in agreement with Liu et al. (2020), who reported that the phenolic content in tea plants increased from immature to mature phase. The increase in phenolic content was due to the fact that plants in the fruit ripening phase produce phenolic compounds as secondary metabolites (Swallah et al., 2020), as well as a function of plant protection against environmental stress (Kumar et al., 2020). The increase in total phenolic content depends on the type of plant observed. In contrast to our results, Refilda et al. (2023) reported a decrease in total phenol content in mature harendong fruit extract compared to immature harendong fruit extract.

Table 3. Effect of fruit maturities on total phenolic content (tpc) of different chili genotypes

Genotypes	Fruit Maturity			Genotype means
	Immature	Intermediate	Mature	
		mg GAE g-1 extract		
IPB 005	13,43^{e}	22,02^{d}	28,98^{d}	21,48^{d}
IPB 374	22,25^{c}	27,42^{c}	33,99^{bc}	27,89^{b}
IPB 367	22,69^{c}	26,25^{c}	39,61^{a}	29,52^{b}
IPB 435	18,60^{cd}	22,04^{d}	33,43^{c}	24,69^{c}
IPB 439	23,15^{c}	31,21^{b}	34,42^{c}	29,59^{b}
IPB 074	16,84^{de}	23,40^{d}	29,70^{d}	23,31^{cd}
IPB 141	32,62^{a}	33,64^{a}	35,43^{b}	33,90^{a}
IPB 363	27,93^{b}	33,02^{ab}	41,22^{a}	34,06^{a}
Fruit Maturity means	22,19^{C}	27,37^{B}	34,60^{A}	

[a-e] Numbers followed by the same letter in the same column are not significantly different according to HSD, 5% level.

The average total phenolic content of IPB 363 was not significantly different from IPB 141. Similar results were found in the IPB 374, IPB 367, and IPB 439 genotypes. IPB 005 had the lowest mean total phenolic content and was statistically significantly different from all genotypes except for the IPB 439. IPB 141 had the highest phenolic content in immature and intermediate fruits but was second in mature fruit. The IPB 363 genotype showed the highest phenolic content of mature chili fruit.

Total phenolic content testing covers all types of phenolics contained therein. Total phenolic content testing was carried out using gallic acid standards. Gallic acid is a component of hydrolyzed tannin compounds (Mahindrakar & Rathod, 2020). The total phenolic content value is expressed in the gallic acid equivalent (GAE). Phenolic compounds commonly found in fruit parts are tannins (Hadi & Taimooz, 2018; Mahindrakar & Rathod, 2020). These compounds protect plants, especially the fruit, from herbivore attacks (Hadi & Taimooz, 2018).

A similar thing happens to the total flavonoid content, increasing from the immature to the mature phase. This is because flavonoids are one of the phenolic compounds found in plants which contain chlorophyll (Aryal et al., 2019). The total flavonoid content (TFC) at the three phases of chili fruit maturity is shown in Table 4. The analysis results on the total flavonoid content showed the same trend as those for total phenolic content. There was an increase in the total flavonoid content from the immature phase to the mature phase. The highest average total flavonoid content was shown by the IPB 363 genotype and was significantly different from those of all other genotypes. The lowest TFC was shown by IPB 074 (2.34 mg QE g^{-1} extract), which was significantly different from IPB 374 (2.62 mg QE g^{-1} extract), IPB 367 (2.52 mg QE g^{-1} extract), IPB 439 (2.60 mg QE g^{-1} extract), and IPB 363 (2.86 mg QE g^{-1} extract). Genotypes that were not significantly different from IPB 074 were: IPB 005 (2.37 mg QE g^{-1} extract), IPB 141 (2.49 mg QE g^{-1} extract), and IPB 435 (2.50 mg QE g^{-1} extract).

Table 4. Effect of fruit maturities on total flavonoid content (tfc) of different chili genotypes

Genotypes	Fruit Maturity			Genotype means
	Immature	Intermediate	Mature	
	mg QE g-1 extract			
IPB 005	1,80^{ab}	2,58^{b}	2,74^{f}	2,37^{d}
IPB 374	1,19^{c}	3,17^{a}	3,48^{cde}	2,62^{b}
IPB 367	1,17^{c}	2,15^{c}	4,24^{a}	2,52^{bc}
IPB 435	1,68^{ab}	2,20^{c}	3,61^{c}	2,50^{bcd}
IPB 439	1,65^{ab}	2,61^{b}	3,54^{cd}	2,60^{b}
IPB 074	1,45^{bc}	2,26^{c}	3,31^{de}	2,34^{d}
IPB 141	1,96^{a}	2,25^{c}	3,24^{e}	2,49^{bcd}
IPB 363	1,53^{bc}	3,16^{a}	3,91^{b}	2,86^{a}
Fruit Maturity means	1,55^{C}	2,55^{B}	3,51^{A}	

$^{a-e}$ Numbers followed by the same letter in the same column are not significantly different according to HSD, 5% level.

The average fruit maturity phase on genotype IPB 363 showed the highest total flavonoid content. Although its total flavonoid content was not the highest when viewed separately by maturity stage, the highest total flavonoid content in immature chili fruit was shown by the IPB 141 genotype, which was not significantly different from IPB 005, IPB

439, and IPB 435. At the intermediate maturity phase, the IPB 363 genotype showed the second-highest results after the IPB 374 genotype. The same results were shown by the mature fruit phase, where IPB 363 showed the second highest results after the IPB 367 genotype.

The antioxidant activity analysis in this study was the DPPH and FRAP methods. Both methods involve different mechanisms. DPPH antioxidants help counteract free radicals in the body while the FRAP antioxidant mechanism helps reduce Fe ions in the body (Parcheta et al., 2021). Mature chili fruit is more optimally used to reduce Fe ions than to counteract free radical compounds. In this study, it was shown that the highest FRAP antioxidants were found in mature chili fruit (Table 5). In contrast, DPPH antioxidants showed high results in immature chili fruit in Table 5.

The highest average DPPH result was found for the IPB 439 genotype (1.76 mg TE g^{-1} extract). However, it was not significantly different from the IPB 074 genotype (1.75 mg TE g^{-1} extract) (Table 5). The lowest DPPH antioxidant activity was found in IPB 374 and IPB 435 genotypes with the same activity values (1.67 mg TE g^{-1} extract). These two showed non-significant differences but were significantly different from the other six genotypes. The highest DPPH antioxidant was based on the fruit maturity level in this study seen in the immature phase. The results for interaction of DPPH and fruit maturity phase revealed that DPPH decreased with fruit maturity. The highest DPPH antioxidant activity at the three fruit maturity phases was shown by different genotypes. Immature fruit with the highest DPPH antioxidant activity was shown by IPB 439. Meanwhile, the highest for intermediate and mature fruits were shown by IPB 435 (1.76 mg TE g^{-1} extract) and IPB 141 (1.66 mg TE g^{-1} extract), respectively.

Table 5. Effect of fruit maturities on the DPPH antioxidant activity of different chili genotypes

Genotypes	Fruit Maturity			Genotype Means
	Immature	Intermediate	Mature	
		µmol TE g-1 extract		
IPB 005	1,88^{c}	1,70^{b}	1,50^{c}	1,69^{d}
IPB 374	1,76^{d}	1,71^{ab}	1,53^{c}	1,67^{e}
IPB 367	1,97^{b}	1,72^{ab}	1,50^{c}	1,73^{bc}
IPB 435	1,79^{d}	1,76^{a}	1,45^{d}	1,67^{e}
IPB 439	2,07^{a}	1,71^{ab}	1,51^{c}	1,76^{a}
IPB 074	1,85^{c}	1,74^{ab}	1,64^{a}	1,75^{ab}
IPB 141	1,80^{d}	1,72^{ab}	1,66^{a}	1,72^{bc}
IPB 363	1,79^{d}	1,74^{ab}	1,59^{b}	1,71^{cd}
Fruit Maturity means	1,86^{A}	1,73^{B}	1,55^{C}	

$^{a-e}$ Numbers followed by the same letter in the same column are not significantly different according to HSD, 5% level.

In contrast to DPPH antioxidant activity, the highest FRAP in this study was shown by the mature phase of chili fruit (46.17 mg TE g^{-1} extract) (Table 6). The lowest FRAP antioxidant activity was shown by immature chili fruit (24.04 mg TE g^{-1} extract) and was significantly different from the other two levels of fruit maturity. The highest average FRAP antioxidant activity was shown by IPB 374 (40.39 mg TE g^{-1} extract), followed by IPB 435, which had the second highest FRAP antioxidant activity (36.45 mg TE g^{-1} extract) and was not significantly different from IPB 439. The lowest average FRAP antioxidant activity was shown by genotype IPB 141 (32.92 mg TE g^{-1} extract). IPB 363 was the genotype that had the lowest FRAP antioxidant activity in immature and intermediate fruit. However, in mature fruit, this genotype showed the highest FRAP (55,61 mg TE g^{-1} extract).

Table 6. Effect of fruit maturity on the antioxidant activity as measured by the FRAP method for different chili genotypes

Genotypes	Fruit Maturity			Genotype Means
	Immature	Intermediate	Mature	
		µmol TE g-1 extract		
IPB 005	22,52^{c}	38,21^{b}	41,97^{e}	34,23^{de}
IPB 374	28,21^{a}	40,64^{a}	52,33^{b}	40,39^{a}
IPB 367	25,18^{b}	32,64^{c}	43,55^{e}	33,79^{ef}
IPB 435	27,36^{a}	33,12^{c}	48,88^{c}	36,45^{b}
IPB 439	23,42^{c}	38,76^{ab}	45,85^{d}	36,01^{bc}
IPB 074	23,97^{bc}	37,30^{b}	39,30^{f}	33,53^{ef}
IPB 141	23,36^{c}	33,48^{c}	41,91^{e}	32,92^{f}
IPB 363	18,27^{d}	31,30^{c}	55,61^{a}	35,06^{cd}
Fruit Maturity means	24,04^{C}	35,68^{B}	46,17^{A}	

$^{a-e}$ Numbers followed by the same letter in the same column are not significantly different according to HSD, 5% level.

The highest α-glucosidase inhibitory activity was found in young chili fruit. In line with the results in this study, Dudoit et al. (2020) found that young grapes had high α-glucosidase inhibitory activity compared to grapes in the mature ripeness phase. In addition to the chilis in the young ripeness phase, high α-glucosidase inhibitory activity was found in ornamental chili IPB 367 (Table 7). This is thought to be related to the purple color of young IPB 367 fruits. The purple color in plants is caused by the content of anthocyanins and total flavonoids (Shi et al., 2020).

The highest AGI was found in the immature chili fruit (54.01%). Statistically, the α-glucosidase inhibitor activity of immature chili fruit was significantly different from intermediate fruit (43.23%) and mature fruit (37.63%). IPB 367, an ornamental chili, had the highest average AGI at all maturity phases. The average AGI of IPB 367 genotype was not significantly different from IPB 435. The lowest average AGI content was shown by IPB 439 (18.67%) and IPB 363 (19.08%) which were large chili and sweet pepper genotypes. The large chili IPB 005 and IPB 374 at the immature fruit phase had an inhibitory activity of more than 50% (68.37 and 62.06%). The AGI content decreased from the immature fruit to the mature fruit, as can be seen in Table 7.

Table 7. Effect of fruit maturity on the α-glucosidase inhibitory activity (AGI) of different chili genotypes

Genotypes	Fruit Maturity			Genotype Means
	Immature	Intermediate	Mature	
			%	
IPB 005	68,37[b]	55,37[b]	48,95[c]	57,56[b]
IPB 374	62,06[c]	53,42[b]	44,00[c]	53,16[c]
IPB 367	75,38[a]	67,08[a]	63,81[a]	68,76[a]
IPB 435	73,60[ab]	65,99[a]	57,98[b]	65,85[a]
IPB 439	36,66[e]	12,78[d]	6,56[d]	18,67[e]
IPB 074	39,16[e]	37,21[c]	34,71[c]	37,03[d]
IPB 141	48,73[d]	36,62[c]	33,25[c]	39,53[d]
IPB 363	28,10[f]	17,34[d]	11,81[d]	19,08[e]
Fruit Maturity means	54,01[A]	43,23[B]	37,63[C]	

[a-e] Numbers followed by the same letter in the same column are not significantly different according to HSD, 5% level.

The chili α-glucosidase inhibitory activity values were divided into three categories: low (<40%), medium (40-60%), and high (>60%) (Getahun et al., 2021). Cardullo et al. (2020) stated that α-glucosidase inhibitory activity of more than 50% has the potential to be used as a functional food to help prevent DM II. The results of this study indicate that IPB 005 and IPB 374 were in the medium group. The high group consisted of the genotypes IPB 367 and IPB 435 while the low group consisted of IPB 363, IPB 439, IPB 141, and IPB 074. Functional biochemical information at three levels of chili fruit ripeness can be directly used as recommendations for chili consumption. In addition, agronomists can better study the physiological responses of chili fruit, especially in secondary metabolites. Chili breeders can use this information to assemble new varieties, especially those that correlate with functional biochemical performance.

4. Conclusions

The highest total phenolic content, total flavonoid content, and FRAP antioxidant activity were found in mature chili fruit. In contrast, α-glucosidase inhibitory activity and DPPH antioxidant activity were shown in young chili fruit. Ornamental chili with young, purple-colored fruit had α-glucosidase inhibitory of more than 75%. This indicates that there was an interaction between anthocyanin compounds and α-glucosidase inhibitory activity. Meanwhile, sweet pepper had the smallest α-glucosidase inhibitory activity. Chili fruit has functional biochemicals that have the potential to help overcome type II diabetes mellitus.

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6. Authors' Contributions

Zulfikar Damaralam Sahid: performed research; wrote the paper; coordinated research; Muhamad Syukur: designed research; performed research; coordinated research; Abdul Qadir: analyzed data; check the paper similarity; analytic tools; Awang Maharijaya: analyzed data; performed PostHoc test; Kharisma Firman Ariyanto: coordinated research; field expertise; Waras Nurcholis: wrote the paper; laboratory analysis; Andi Nadia Nurul Lathifa Hatta: wrote the paper; checked final submission.

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7. Conflicts of Interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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