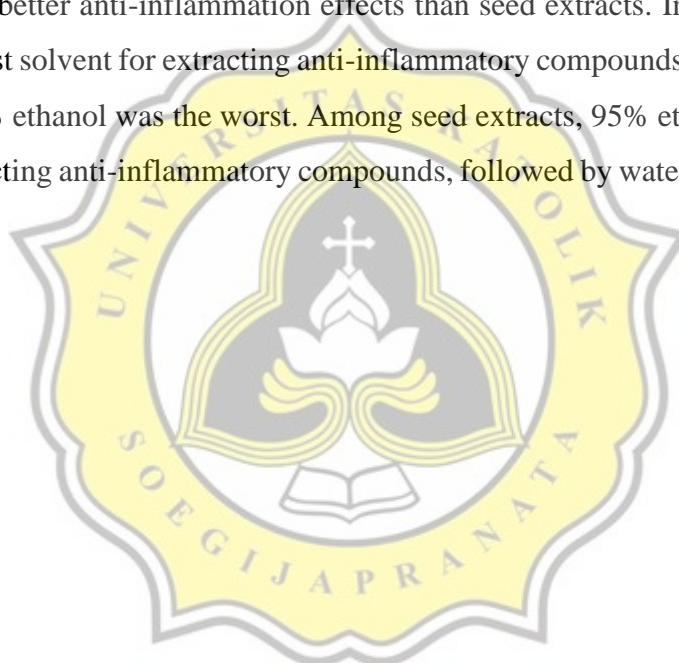


## 4. Results

### 4.1. Nitric Oxide Determination

LPS induced zebrafish embryos to produce inflammatory symptoms. The amount of NO produced in the LPS-induced group was higher than that of the control group (Figure 4 and Table 2). L-NMMA, (N<sup>5</sup>-[imino(methylamino)methyl]-L-ornithine, a clinical drug used to treat inflammation, effectively reduced the number of macrophages. Compared with the LPS-induction group, all tested samples not only reduced the production of NO, but also showed a dose-dependent inhibitory effect (Figure 4A-F and Table 2). Taking NO concentration as an indicator of inflammation, 1% water extract of the peel showed the strongest inhibitory effect on LPS-induced inflammation ( $p < 0.05$ ). Generally, peel extracts showed better anti-inflammation effects than seed extracts. In the peel extracts, water was the best solvent for extracting anti-inflammatory compounds, followed by 50% ethanol, and 95% ethanol was the worst. Among seed extracts, 95% ethanol was the best solvent for extracting anti-inflammatory compounds, followed by water, and 50% ethanol is the worst.



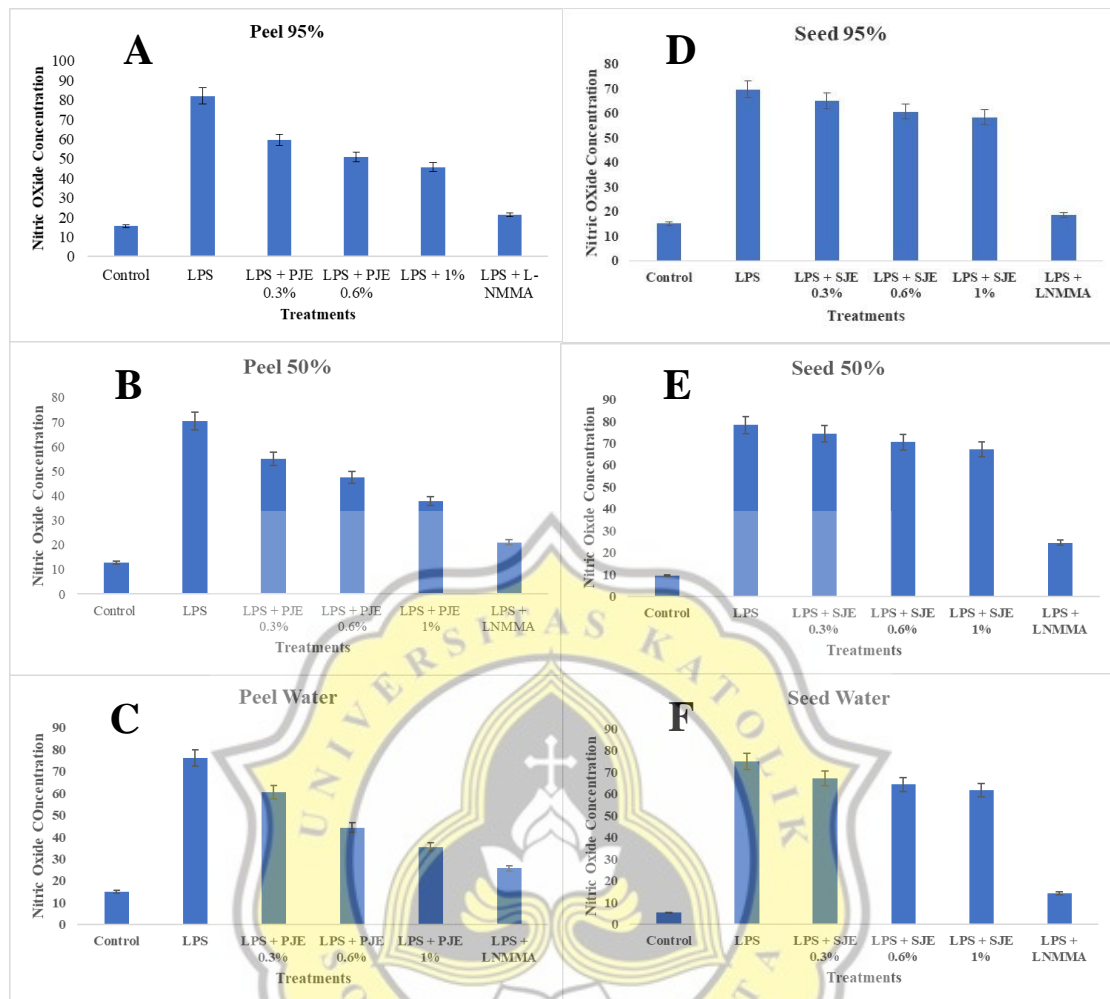


Figure 4. Determination of nitric oxide content in zebrafish with treatments of : (A) Jaboticaba peel ethanol extract 95%, (B) Jaboticaba peel ethanol extract 50%, (C) Jaboticaba peel water extract and (D) Jaboticaba seed ethanol extract 95%, (E) Jaboticaba seed ethanol extract 50%, (F) Jaboticaba seed water extract. n =8

Table 2. Nitric Oxide Determination in Zebrafish with Various Treatments of Jaboticaba

Treatments	Nitric Oxide Concentrations ( $\mu\text{M}$ )					
	Peel 95% EtOH	Peel 50% EtOH	Peel Water	Seed 95% EtOH	Seed 50% EtOH	Seed Water
Control	15.542 $\pm$ 0.62 <sup>a</sup>	12.746 $\pm$ 0.75 <sup>a</sup>	15.018 $\pm$ 0.41 <sup>a</sup>	15.225 $\pm$ 0.58 <sup>a</sup>	9.503 $\pm$ 0.14 <sup>a</sup>	5.309 $\pm$ 0.43 <sup>a</sup>
LPS	82.111 $\pm$ 0.32 <sup>f</sup>	70.406 $\pm$ 0.74 <sup>f</sup>	76.080 $\pm$ 0.47 <sup>f</sup>	69.836 $\pm$ 0.99 <sup>f</sup>	78.403 $\pm$ 0.55 <sup>f</sup>	74.938 $\pm$ 0.60 <sup>f</sup>
LPS + JE 0.3%	59.627 $\pm$ 0.84 <sup>eB</sup>	55.056 $\pm$ 0.71 <sup>eA</sup>	60.445 $\pm$ 0.14 <sup>eB</sup>	65.086 $\pm$ 0.63 <sup>eC</sup>	74.314 $\pm$ 0.30 <sup>eE</sup>	67.222 $\pm$ 0.64 <sup>eD</sup>
LPS + JE 0.6%	50.935 $\pm$ 0.80 <sup>dC</sup>	47.394 $\pm$ 0.92 <sup>dB</sup>	44.310 $\pm$ 0.32 <sup>dA</sup>	60.781 $\pm$ 0.50 <sup>dD</sup>	70.572 $\pm$ 0.52 <sup>dF</sup>	64.321 $\pm$ 0.21 <sup>dE</sup>
LPS + JE 1%	45.673 $\pm$ 1.69 <sup>cC</sup>	37.863 $\pm$ 0.05 <sup>cB</sup>	35.490 $\pm$ 0.22 <sup>cA</sup>	58.503 $\pm$ 0.42 <sup>cD</sup>	67.333 $\pm$ 0.33 <sup>cF</sup>	61.636 $\pm$ 1.18 <sup>cE</sup>
LPS + LNMMA	21.327 $\pm$ 0.06 <sup>b</sup>	20.962 $\pm$ 1.60 <sup>b</sup>	25.755 $\pm$ 0.10 <sup>b</sup>	18.697 $\pm$ 0.42 <sup>b</sup>	24.535 $\pm$ 0.43 <sup>b</sup>	14.259 $\pm$ 0.25 <sup>b</sup>

<sup>a-f</sup>Different letters in the same column shows significant difference between treatments ( $p < 0.05$ ).

<sup>A-F</sup>Different letters in the same row shows significant difference between treatments ( $p < 0.05$ ).

JE = Jaboticaba Extract

n = 8

## 4.2. Macrophage Determination

LPS treatment increased the production of macrophages in zebrafish, indicated by the increase of light intensity (Figure 5). The number of macrophages in the L-NMMA group is close to the control group (Figure 6A-F and Table 3). Treatment with jaboticaba extract tended to attenuate the increase in macrophage production induced by LPS, generally in a dose-dependent manner. At the same dose, the inhibitory effects of different extracts on macrophages had no significant difference ( $p>0.05$ ).



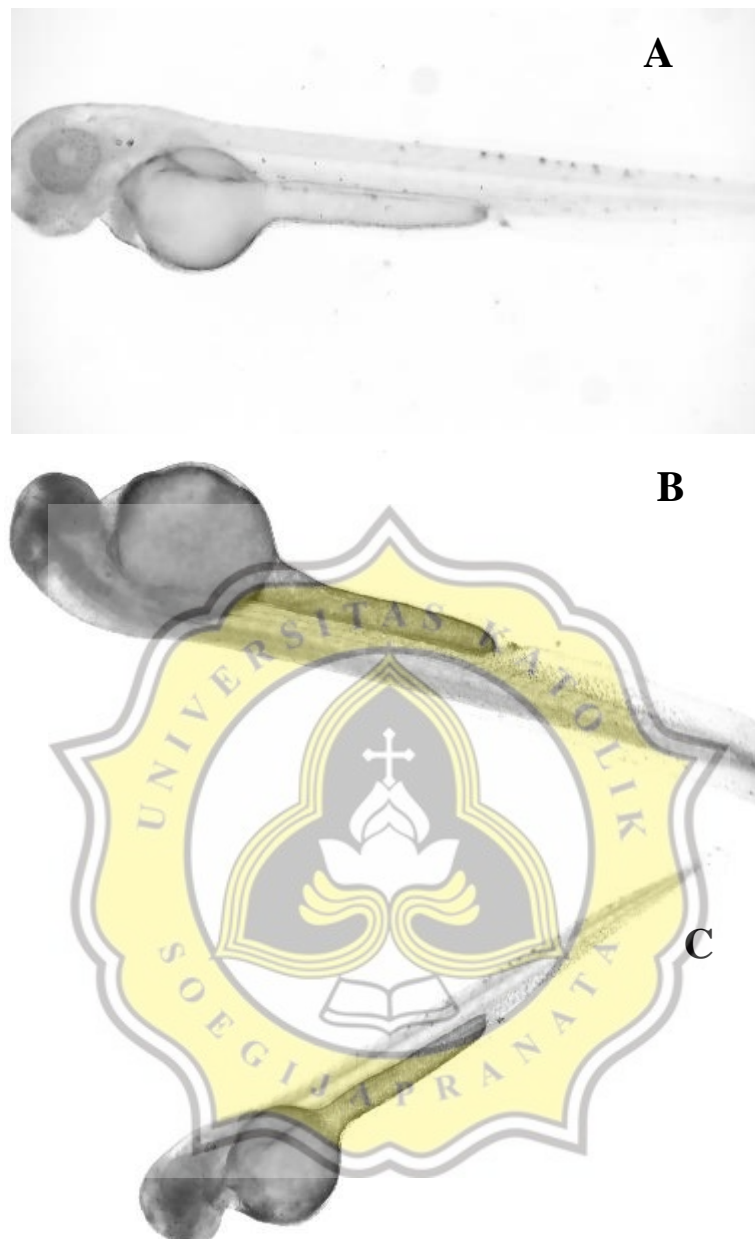


Figure 5. Representative pictures of neutral red stained zebrafish larvae (A = Control, B = LPS induced, C = LPS+Jaboticaba Extract)



Figure 6. Determination of macrophages in zebrafish with treatments of : (A) Jaboticaba peel ethanol extract 95%, (B) Jaboticaba peel ethanol extract 50%, (C) Jaboticaba peel water extract and (D) Jaboticaba seed ethanol extract 95%, (E) Jaboticaba seed ethanol extract 50%, (F) Jaboticaba seed water extract.  $n = 8$

Table 3. Effect of Jaboticaba on Macrophage Staining of Zebrafish

Treatments	Intensity (pixel)					
	Peel 95% EtOH	Peel 50% EtOH	Peel Water	Seed 95% EtOH	Seed 50% EtOH	Seed Water
Control	145,322±3253 <sup>a</sup>	151,308±1960 <sup>a</sup>	144,916±2524 <sup>a</sup>	172,816±3927 <sup>a</sup>	156,656±3978 <sup>a</sup>	156,032±8198 <sup>a</sup>
LPS	236,711±8849 <sup>f</sup>	208,362±1155 <sup>d</sup>	208,189±2652 <sup>d</sup>	193,803±3059 <sup>c</sup>	208,956±9636 <sup>d</sup>	209,596±7851 <sup>d</sup>
LPS + JE 0.3%	198,630±6847 <sup>e</sup>	178,625±4791 <sup>bc</sup>	186,188±5522 <sup>c</sup>	190,725±4070 <sup>c</sup>	197,363±7130 <sup>c</sup>	199,390±9145 <sup>d</sup>
LPS + JE 0.6%	187,432±1794 <sup>d</sup>	183,670±6910 <sup>c</sup>	175,550±502 <sup>c</sup>	175,383±3166 <sup>ab</sup>	199,862±6806 <sup>cd</sup>	189,309±6697 <sup>c</sup>
LPS + JE 1%	173,575±1918 <sup>c</sup>	173,888±892 <sup>b</sup>	178,608±9769 <sup>c</sup>	177,318±5084 <sup>ab</sup>	185,638±2396 <sup>b</sup>	177,203±3803 <sup>b</sup>
LPS + LNMMA	158,254±5224 <sup>b</sup>	157,048±8063 <sup>a</sup>	157,650±8975 <sup>b</sup>	172,816±5224 <sup>a</sup>	184,863±3634 <sup>b</sup>	168,977±2260 <sup>b</sup>

<sup>a-f</sup>Different letters show significant difference between treatments ( $p < 0.05$ ).

JE = Jaboticaba Extract; L-NMMA: N<sup>5</sup>-[imino(methylamino)methyl]-L-ornithine

n = 8





### 4.3. Neutrophils Determination

#### 4.3.1. Individual Size

Figure 7-10 and Table 4-9 shows the results of neutrophil determination using Sudan Black staining. Compared to the control, LPS induced inflammatory symptoms in zebrafish embryos, manifested by an increase in the size of individual neutrophils (Figure 8 and Table 4). Treatment with 0.6% of 95% ethanol extract of peel showed the strongest inhibitory effect on LPS-induced inflammation ( $p < 0.05$ ). Peel extracts showed better effect on reduce the size of neutrophils than seed extracts. At the highest concentration applied (1%), there is no significant difference between peel extracted with ethanol 95%, ethanol 50% and water ( $p < 0.05$ , Table 5). Seed extracts did not show a positive effect in reducing the size of neutrophils, and even increased the size of neutrophils.

#### 4.3.2. Total Number of Neutrophil

LPS induction increased the total number of neutrophils (Figure 9 and Table 6). 1% water seed extract showed the strongest inhibitory effect on LPS-induced inflammation ( $p < 0.05$ ). For this parameter, seed extracts showed better anti-inflammation effects than peel extracts in general. When the total number of neutrophils was used as the inflammatory biomaker, 95% ethanol was the best solvent in peel extracts, followed by 50% ethanol, and water extracts are the worst. In seed extracts, 95% ethanol and 50% ethanol were the best solvent for reducing production of neutrophils, and water is the worst. At the highest concentration applied (1%), there is no significant difference among 95% ethanol extract of peel and seed, and 50% ethanol extract of seed ( $p < 0.05$ , Table 7).

#### 4.3.3. Total Size of Neutrophil

LPS induced zebrafish embryos to produce inflammatory symptoms, so the total size of neutrophils increased significantly, ( $p < 0.05$ , Figure 10 and Table 8). Compared with the LPS-induction group, all tested samples not only reduced the total size of neutrophils, but also showed a dose-dependent inhibitory effect. Taking total size of neutrophils as an indicator of inflammation, 1% water extract of the peel showed the strongest inhibitory effect of LPS-induced inflammation ( $p < 0.05$ ). Generally, peel extracts showed better anti-inflammation effects than seed extracts. In the peel extracts, ethanol 95% was the best solvent for extracting anti-inflammatory compounds, followed by 50% ethanol, and water was the worst. Among seed extracts, 95% ethanol was the best solvent for extracting anti-inflammatory compounds, followed by water and 50% ethanol.



When zebrafish embryos were treated with 1% of extracts, 95% ethanol extract of peel group had the lowest total size of Neutrophils, which showed the highest inhibition, and the water extract of peel had the lowest inhibition ( $p < 0.05$ , Table 9). The 50% ethanol extract of peel showed no significant difference with seed extracts, no matter which solvent was used to extract seeds ( $p > 0.05$ ).





Figure 7. Representative pictures of Sudan Black stained zebrafish larvae (A = Control, B = LPS induced, C = LPS+Jaboticaba Extract)

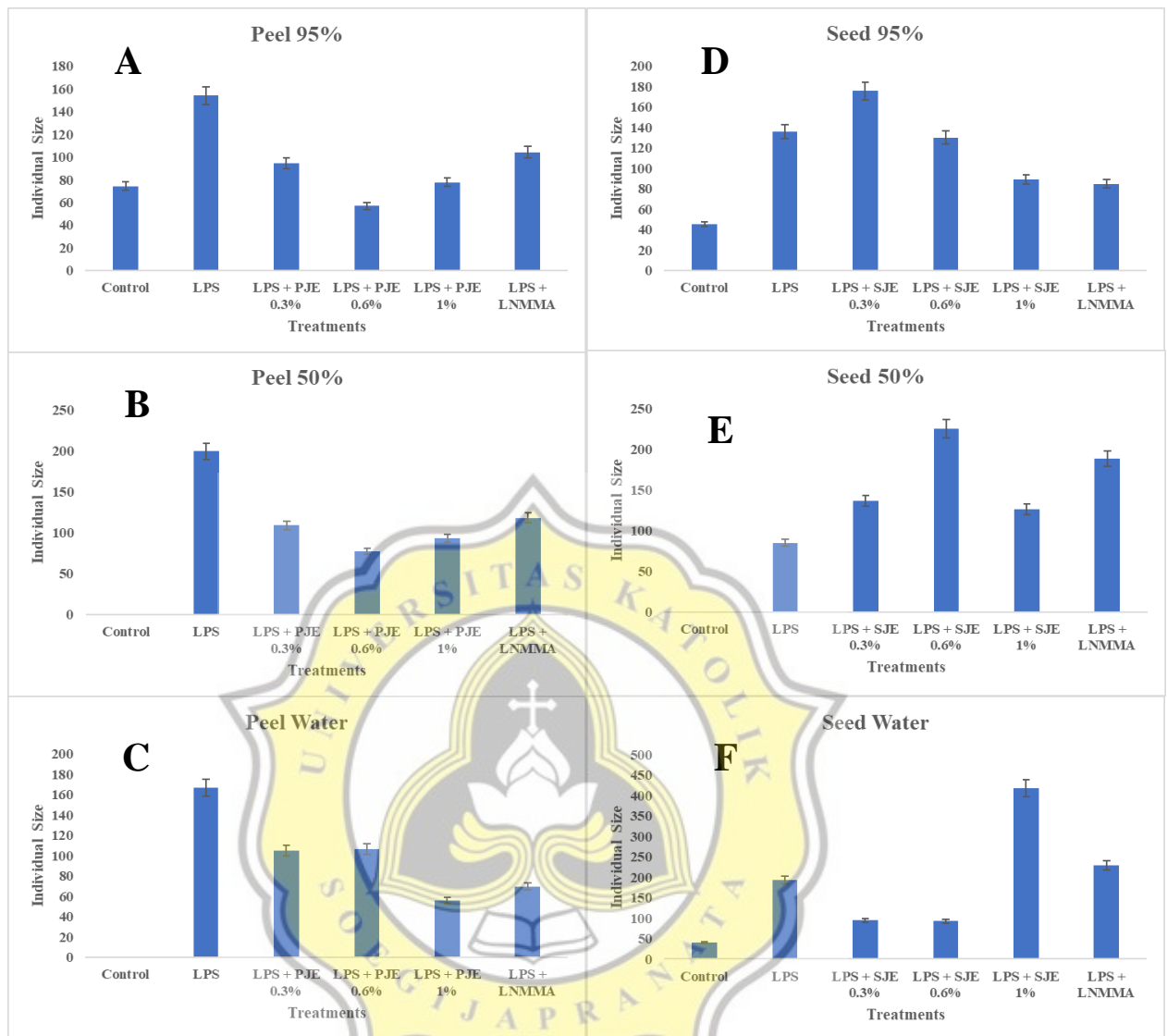


Figure 8. Evaluation of neutrophils individual size in zebrafish with treatments of : (A) Jaboticaba peel ethanol extract 95%, (B) Jaboticaba peel ethanol extract 50%, (C) Jaboticaba peel water extract and (D) Jaboticaba seed ethanol extract 95%, (E) Jaboticaba seed ethanol extract 50%, (F) Jaboticaba seed water extract. n=8

Table 4. Effects of Jaboticaba on Individual Size of Zebrafish Neutrophils

Treatments	Individual Size (pixel)					
	Peel 95% EtOH	Peel 50% EtOH	Peel Water	Seed 95% EtOH	Seed 50% EtOH	Seed Water
Control	74.27±1.18 <sup>b</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	45.55±4.91 <sup>a</sup>	0.00±0.00 <sup>a</sup>	40.93±3.41 <sup>a</sup>
LPS	153.93±0.83 <sup>e</sup>	199.57±8.65 <sup>f</sup>	167.05±7.53 <sup>e</sup>	135.68±6.56 <sup>c</sup>	84.73±3.48 <sup>b</sup>	193.55±6.20 <sup>c</sup>
LPS + JE 0.3%	94.61±0.32 <sup>cA</sup>	108.99±5.21 <sup>dB</sup>	104.91±3.85 <sup>dB</sup>	175.60±3.65 <sup>dD</sup>	136.40±1.62 <sup>dC</sup>	94.51±2.83 <sup>bA</sup>
LPS + JE 0.6%	56.93±1.30 <sup>aA</sup>	76.99±0.94 <sup>bB</sup>	106.72±0.79 <sup>dD</sup>	130.06±9.90 <sup>cE</sup>	225.31±5.45 <sup>fF</sup>	92.35±2.09 <sup>bC</sup>
LPS + JE 1%	77.76±0.62 <sup>bB</sup>	93.33±5.88 <sup>cB</sup>	55.98±6.48 <sup>bA</sup>	89.26±5.24 <sup>bB</sup>	126.06±5.14 <sup>cC</sup>	418.42±19.20 <sup>eD</sup>
LPS + LNMMA	104.27±4.54 <sup>d</sup>	118.32±0.30 <sup>e</sup>	69.88±13.03 <sup>c</sup>	84.71±4.16 <sup>b</sup>	188.26±0.57 <sup>e</sup>	229.38±7.57 <sup>d</sup>

<sup>a-f</sup>Different letters in the same column shows significant difference between treatments ( $p < 0.05$ ).

<sup>A-F</sup>Different letters in the same row shows significant difference between treatments ( $p < 0.05$ ).

JE = Jaboticaba Extract.

n = 8

Table 5. Percentage Inhibition of Jaboticaba Extract in Individual Size of Neutrophils

Type of Extract (1%)	% Inhibition in Individual Size of Neutrophils
Peel 95% EtOH	50.34±0.01 <sup>d</sup>
Peel 50% EtOH	53.25±0.04 <sup>d</sup>
Peel Water	46.58±0.02 <sup>d</sup>
Seed 95% EtOH	2.71±0.06 <sup>c</sup>
Seed 50% EtOH	-92.14±0.10 <sup>a</sup>
Seed Water	-4.34±0.13 <sup>b</sup>

<sup>a-d</sup>Different letters in the same column shows significant difference between treatments (p < 0.05).

n=8

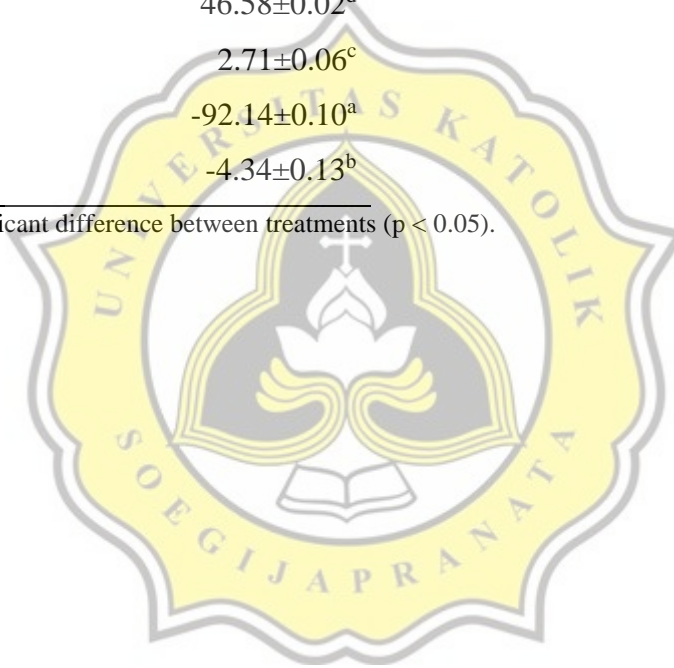




Figure 9. Evaluation of neutrophils total number in zebrafish with treatments of : (A) Jaboticaba peel ethanol extract 95%, (B) Jaboticaba peel ethanol extract 50%, (C) Jaboticaba peel water extract and (D) Jaboticaba seed ethanol extract 95%, (E) Jaboticaba seed ethanol extract 50%, (F) Jaboticaba seed water extract. n=8



Table 6. Effect of Jaboticaba on Total Number of Zebrafish Neutrophils

Treatments	Total Number					
	Peel 95% EtOH	Peel 50% EtOH	Peel Water	Seed 95% EtOH	Seed 50% EtOH	Seed Water
Control	9.33±0.58 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	8.67±0.58 <sup>a</sup>	0.00±0.00 <sup>a</sup>	4.33±0.58 <sup>a</sup>
LPS	35.33±5.86 <sup>d</sup>	14.00±0.00 <sup>d</sup>	12.67±3.21 <sup>b</sup>	47.00±4.58 <sup>c</sup>	26.00±1.73 <sup>f</sup>	16.67±3.06 <sup>b</sup>
LPS + JE 0.3%	15.00±1.00 <sup>bcA</sup>	24.33±2.08 <sup>dC</sup>	21.67±4.16 <sup>cdBC</sup>	17.33±3.79 <sup>bBC</sup>	15.00±1.00 <sup>eA</sup>	14.67±3.21 <sup>bA</sup>
LPS + JE 0.6%	16.67±3.06 <sup>dB</sup>	14.33±0.58 <sup>dB</sup>	25.67±2.31 <sup>dC</sup>	18.00±3.00 <sup>bB</sup>	8.00±1.73 <sup>cA</sup>	23.33±2.08 <sup>cC</sup>
LPS + JE 1%	10.67±0.58 <sup>abB</sup>	9.00±3.00 <sup>bcB</sup>	20.00±4.36 <sup>cC</sup>	18.00±2.00 <sup>bC</sup>	10.67±0.58 <sup>dB</sup>	2.67±0.58 <sup>aA</sup>
LPS + LNMMA	10.67±1.53 <sup>ab</sup>	167.31±0.58 <sup>cd</sup>	8.67±1.15 <sup>b</sup>	14.67±0.58 <sup>b</sup>	33.33±1.53 <sup>b</sup>	3.00±0.00 <sup>a</sup>

<sup>a-f</sup>Different letters in the same column shows significant difference between treatments ( $p < 0.05$ ).

<sup>A-F</sup>Different letters in the same row shows significant difference between treatments ( $p < 0.05$ ).

JE = Jaboticaba Extract

n=8

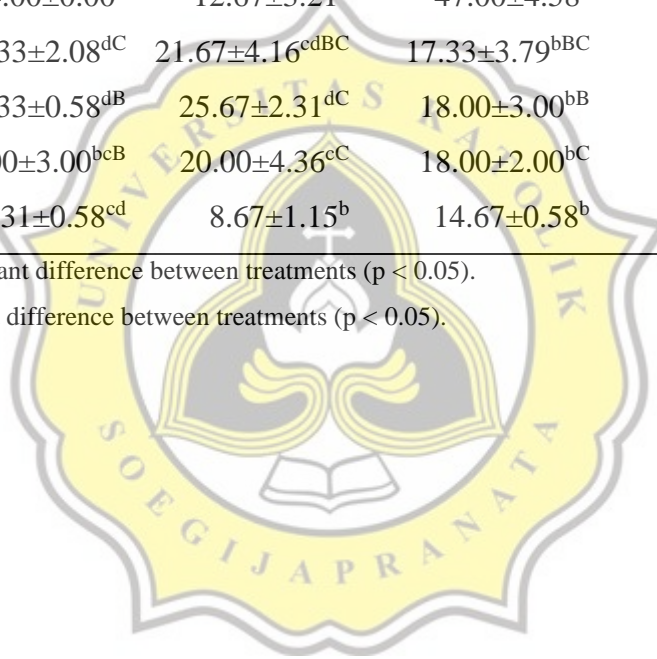
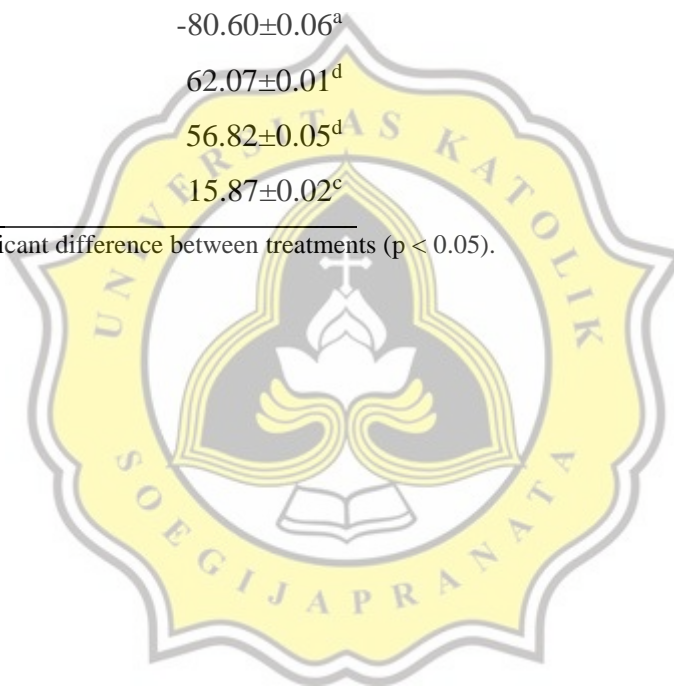


Table 7. Percentage Inhibition of Jaboticaba Extract in Total Number of Neutrophils

Type of Extract (1%)	% Inhibition in Total Number of Neutrophils
Peel 95% EtOH	59.50±0.06 <sup>d</sup>
Peel 50% EtOH	-11.29±0.04 <sup>b</sup>
Peel Water	-80.60±0.06 <sup>a</sup>
Seed 95% EtOH	62.07±0.01 <sup>d</sup>
Seed 50% EtOH	56.82±0.05 <sup>d</sup>
Seed Water	15.87±0.02 <sup>c</sup>

<sup>a-d</sup>Different letters in the same column shows significant difference between treatments (p < 0.05).

n=8



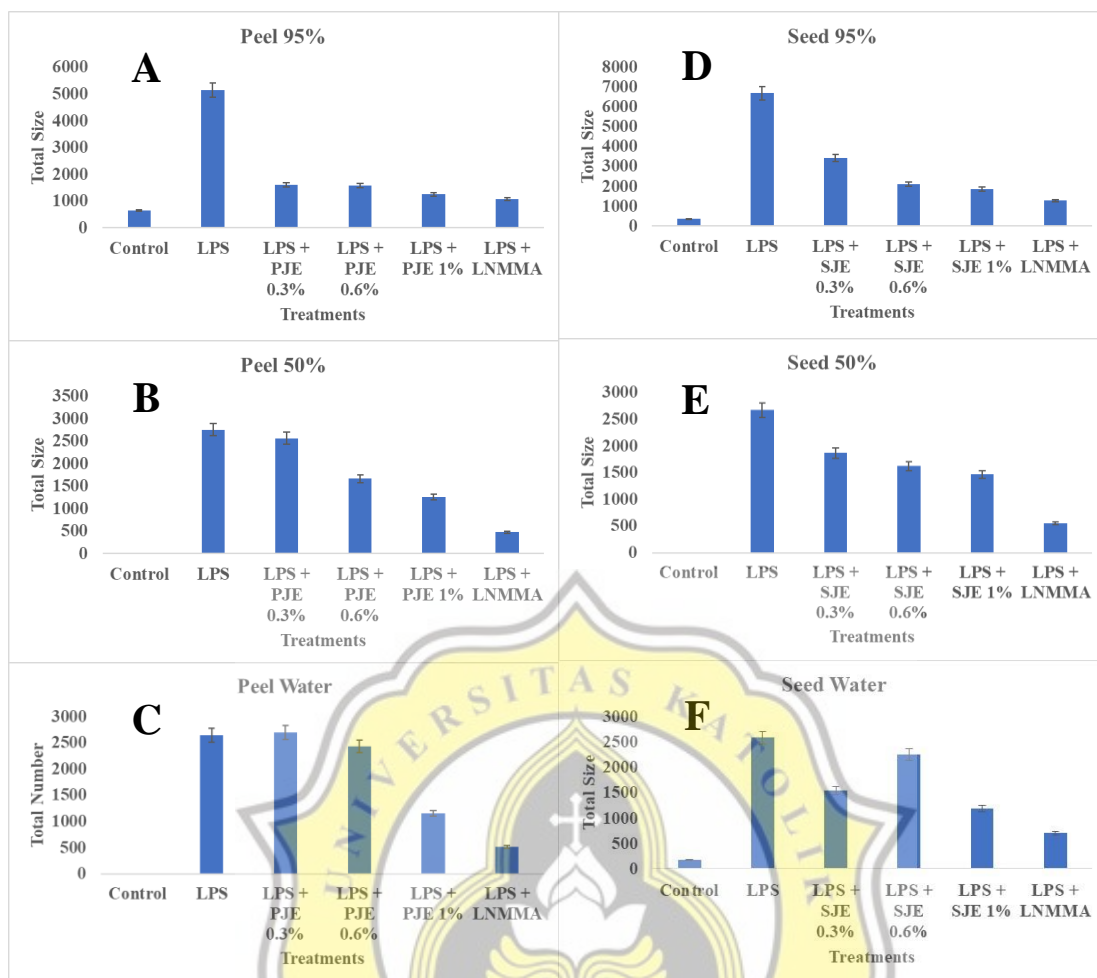


Figure 10. Evaluation of neutrophils total size in zebrafish with treatments of : (A) Jaboticaba peel ethanol extract 95%, (B) Jaboticaba peel ethanol extract 50%, (C) Jaboticaba peel water extract and (D) Jaboticaba seed ethanol extract 95%, (E) Jaboticaba seed ethanol extract 50%, (F) Jaboticaba seed water extract. n=8

Table 8. Effect of Jaboticaba on Total Size of Zebrafish Neutrophils

Treatments	Total Size (pixel)					
	Peel 95% EtOH	Peel 50% EtOH	Peel Water	Seed 95% EtOH	Seed 50% EtOH	Seed Water
Control	653.00±11.27 <sup>a</sup>	0.00±0.00 <sup>a</sup>	0.00±0.00 <sup>a</sup>	362.67±20.01 <sup>a</sup>	0.00±0.00 <sup>a</sup>	176.33±3.79 <sup>a</sup>
LPS	5150.67±35.39 <sup>e</sup>	2754.00±46.89 <sup>f</sup>	3132.00±3.61 <sup>c</sup>	6674.67±175.91 <sup>f</sup>	2667.67±26.76 <sup>f</sup>	2573.00±56.31 <sup>f</sup>
LPS + JE 0.3%	1608.00±81.43 <sup>dA</sup>	2564.00±91.11 <sup>eC</sup>	2697.00±58.92 <sup>cD</sup>	3417.00±90.32 <sup>eE</sup>	1867.33±42.10 <sup>eB</sup>	1540.00±31.56 <sup>dA</sup>
LPS + JE 0.6%	1578.00±76.39 <sup>dA</sup>	1662.67±88.93 <sup>dA</sup>	2434.00±11.53 <sup>bcC</sup>	2126.67±64.08 <sup>dB</sup>	1620.00±73.00 <sup>dA</sup>	2239.67±25.53 <sup>eB</sup>
LPS + JE 1%	1254.33±69.17 <sup>cAB</sup>	1257.00±59.09 <sup>cAB</sup>	1133.00±16.26 <sup>abA</sup>	1864.33±36.25 <sup>cC</sup>	1464.67±13.05 <sup>cB</sup>	1178.67±22.05 <sup>cA</sup>
LPS + LNMMA	1065.00±26.51 <sup>b</sup>	476.33±38.68 <sup>b</sup>	615.33±34.27 <sup>a</sup>	1281.67±52.17 <sup>b</sup>	553.67±60.54 <sup>b</sup>	699.67±7.64 <sup>b</sup>

<sup>a-f</sup>Different letters in the same column shows significant difference between treatments ( $p < 0.05$ ).

<sup>A-F</sup>Different letters in the same row shows significant difference between treatments ( $p < 0.05$ ).

JE = Jaboticaba Extract,

n=8

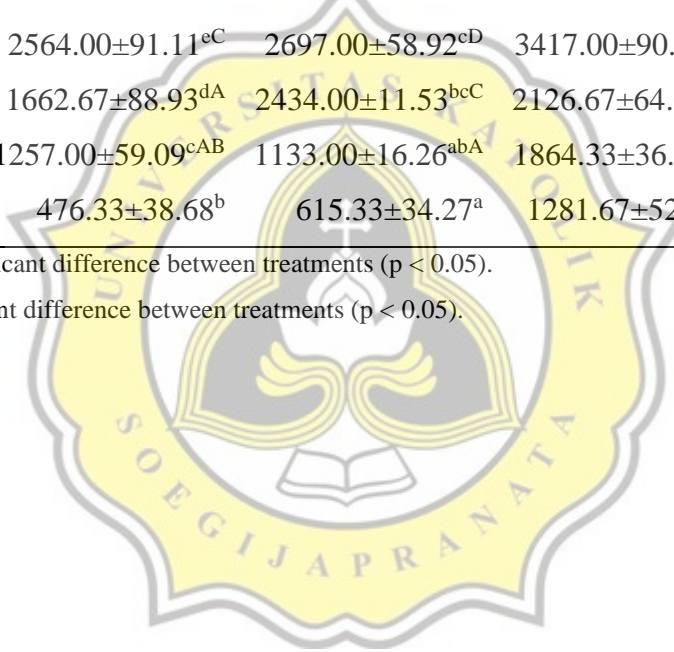


Table 9. Percentage Inhibition of Jaboticaba Extract in Total Size of Neutrophils

Type of Extract (1%)	% Inhibition in Total Size of Neutrophils
Peel 95% EtOH	95.88±0.03 <sup>c</sup>
Peel 50% EtOH	33.62±0.02 <sup>ab</sup>
Peel Water	20.70±0.01 <sup>a</sup>
Seed 95% EtOH	62.97±0.00 <sup>b</sup>
Seed 50% EtOH	38.11±0.01 <sup>ab</sup>
Seed Water	35.74±0.01 <sup>ab</sup>

<sup>a-c</sup>Different letters in the same column shows significant difference between treatments (p < 0.05).

n=8

