RESEARCH ARTICLE

Effects of NADPH oxidase inhibitor of the *Etlingera elatior* (*E. elatior*) fruits extracts in animal sepsis models

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ABSTRACT

Infectious diseases with complications of sepsis are still public health concern in both developed and developing countries. Sepsis is a potentially life-threatening systemic immune response to infection that can lead to end- stage organ failure and death. Sepsis involves multiple mechanisms such as neuroendocrine, complement activation, blood coagulation, and fibrinolytic system. Reactive oxygen species (ROS) is an inflammatory mediator produced by NADPH oxidase activation. This study aimed to investigate the effects of ethanol extract of *E. elatior* fruits on NADPH oxidase activity. Forty *Mus musculus* mice were randomized divided into five groups (n=8), with the intervention group receiving an intraperitoneal injection of 0.3 mg/kg BW lipopolysaccharide (LPS). There was a normal group without LPS injection (N-1), LPS injection only (N-2), and those that received LPS injection and ethanol extracts of *E. elatior* fruits containing 2.1 mg/20 g (N-3), 4.2 mg/20 g (N-4), and 8.4 mg/20 g (N-5). NADPH oxidase activity were measured using ELISA. The oneway ANOVA was used to investigate the differences between the groups. After administration of the extract at a varied dose, N-5 group the lowest NADPH oxidase activity (p=0.001). The ethanol extract of *E. elatior* fruit has antioxidant effects. In this study, a dose of 8.4 mg/20 g of extract significantly reduced NADPH oxidase activity. The ethanol extract of *E. elatior* might be considered a treatment in sepsis.

Keywords: Sepsis; NADPH oxidase; *E. elatior*.

INTRODUCTION

Sepsis affects millions of people each year and has a high morbidity and mortality rates (Septimus, 2020). Sepsis is characterized by oxidative damage and multiorgan dysfunction. It is caused by an infection followed by an uncontrolled inflammatory response (Singer *et al.*, 2016). Septic shock is defined as the sepsis group with severe disturbances in circulation and cellular metabolism in patients. Septic shock is characterized by hypotension despite adequate fluid resuscitation or the need for vasopressors to maintain mean arterial pressure (MAP) $\geq$ 65 mm Hg or lactate levels greater than 2 mmol/L (Rhodes *et al.*, 2017).

Reactive oxygen species (ROS) production will increase as a result of the proinflammatory response during infection (Lopes *et al.*, 2022). The physiological function of ROS are defense, second messenger, control of proinflammatory signaling, profibrotic signaling, cell proliferation, apoptosis and other activities without causing macromolecular damage (Checa & Aran, 2020). However, sepsis causes an excess production of ROS which can lead to damage cells and organs (Lopes *et al.*, 2022). ROS production can increase the severity of sepsis and causes damage vital organs such

as liver, kidney, heart and colon (Meng & Zhang, 2019). In sepsis, ROS is the main etiology of endothelial dysfunction (Diebold *et al.*, 2015). ROS consist of superoxide anion (o^{2-}), nitric oxide radical (NO), hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2) (Joshi & Khan, 2019). There are several sources of ROS such as xanthine oxidase, cyclooxygense, nitric oxide synthase (NOS), peroxidase and nicotinamide adenine dinucleotide (NAPDH) oxidase (Meo *et al.*, 2016). NADPH oxidase is the primary source of ROS (Moghadam *et al.*, 2021).

NADPH oxidase act as a catalyst in the formation of superoxide anion radicals and generates ROS, which lead to oxidative stress and macromolecular damage (Joshi & Khan, 2019). ROS released by the NADPH oxidase complex can activate granular proteases and induce the formation of neutrophil extracellular traps (NETs) and promote the production of the pro-inflammatory cytokines like tumor necrosis factor alpha and macrophage inflammatory protein 2 (MIP-2) (Nguyen *et al.*, 2017). ROS generated by NADPH oxidase will also enhance adhesion proteins and induce Inducible *nitric oxide synthase* (iNos), which can damage microvascular function in sepsis. NADPH oxidase is a source of intracellular ROS which can potentially be as a target for antioxidant therapy (Lopes *et al.*, 2022).
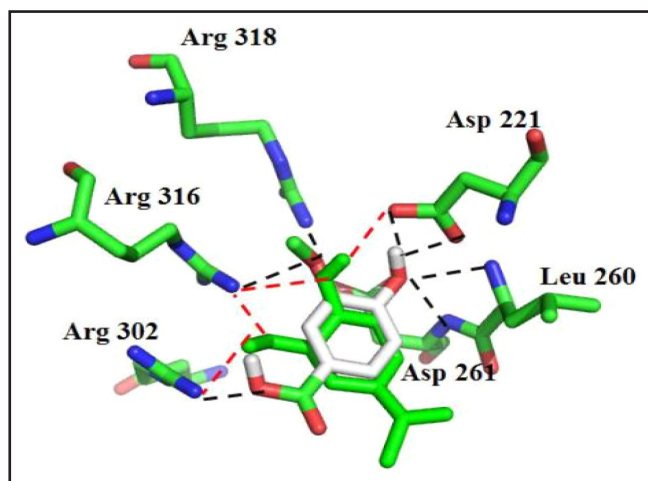
Figure 1. Visualization of binding complex between NADPH oxidase and *Vanillic acid* (Laksono, 2020).

According to biocomputation study, an Indonesian herbal plant component has the potential to inhibit NADPH oxidase via p47-phox. The compound is *Vanillic acid* (VA) (Laksono, 2020). Figure 1 shows that VA has average bond energy with p47-phox of -6,2 kcl/mol that bind to the binding site Asp [221], Leu [260], Asp [261], Arg [302], Arg [316] (Laksono, 2020). VA (4-hydroxy-3-methoxybenzoic acid) is a derivative of hydroxylated phenolic compounds from benzoic and cinnamic acid (Kumar *et al*., 2011). VA is commonly used as a flavoring agent in the food industry. The pharmacological effect of VA includes liver protection (Itoh *et al*., 2009), anti-inflammatory (Prince *et al*., 2011), anti-asthmatic and anti-hypertensive (Kumar *et al*., 2011). According to a recent study on the effects of VA in oxidative liver injury that induced sepsis in rat models, a dose 100 mg/ Kg BW significantly increased levels of glutathione (GSH), glutathione peroxidase (Gpx), superoxide dismutase (SOD) and catalase (CAT) compared to the control and placebo groups (Meng & Zhang, 2019). *Etlingera elatior* (*E. elatior*) has been reported to have a VA component (Sahidin *et al*., 2019). *E. elatior*, locally known as kecombrang is commonly found in Indonesia (Anzian *et al*., 2017). Among bioactive compounds found in *E. elatior* fruit include alkaloids, flavonoids, tanins, and terpenoids (Leorita *et al*., 2018). *E. elatior* also contains micronutrient components that are advantageous for health (Wijekoon *et al*., 2011). These micronutrients function act as antioxidants and boost the immune system (Prasetiyo & Nasronudin, 2015).

The current management of sepsis is not targeted specifically on to inflammation and oxidative stress. Effective adjuvant therapy is needed to reduce inflammation and oxidative stress (Lin *et al*.,

2018). A study *in vivo* is required to examine the effects of *E. elatior* based on the findings of the in biocomputation study. Hence, the purpose to prove that the ethanol extract of *E. elatior* fruits may reduce the activity of NADPH oxidase.

MATERIALS AND METHODS

The male mice of the subspecies *Mus musculus* Balb/C strain were provided by the Faculty of Veterinary Medicine Universitas Gadjah Mada. All stages of research experiments followed animal ethics and the research protocol got permitted from the Health Research Ethics Committee, Dr. Moewardi General Hospital number: 477/ IV/ HREC/ 2021. The *E. elatior* fruits used in this study were purchased from a local farmer in Langkaplancar village, Pangandaran district, west Java Province, Indonesia. Determination of plant species was carried out by the Faculty of Biology Muhammadiyah University of Surakarta with the name *E. elatior* (Jack) from the Zingiberaceae family plant. The VA standard was purchased from Sigma Aldrich.

Extract of *E. elatior* fruits
E. elatior fresh fruit weighing 400 grams was washed and dried. *E. elatior* dried fruits were crushed into a powder. The maceration procedure can be sped up by powder texture. The material was macerated in ethanol 70% for 24 hours (Farida & Maruzy, 2016). The filtrate from maceration process was evaporated using rotary evaporator until it became a thick extract (Leorita *et al*., 2018). High performance liquid chromatography (HPLC) analysis was used to determine the VA components in the ethanol extract of *E. elatior* as shown in figure 3. *Vanillic acid* standard chromatogram is shown in Figure 2.

Experimental design
The study used a post-test only control group design. Forty male *Mus musculus* Balb/C strain aged 3-4 months old, weighing 20-30 g, were obtained from the Faculty of Veterinary Medicine Universitas Gadjah Mada. The mice were adapted in a controlled environment with a 12 hours cycle of light and dark at room temperature. The mice were given BR 1 standard feed with the amount of feed adjusted to their average body weigh while drinking was given freely (ad libitum). The mice were randomly divided into 5 groups (n=8/group): normal mice (N-1), negative control (N-2) and treatment groups (N3-N5). The normal mice (N-1) was given no treatment while negative control (N-2) and treatment groups (N3-N5) received an intraperitoneal lipopolysaccharide (LPS) 0.3 mg/kg BW to induce sepsis (Arifin, 2021). The N-3, N-4, N5 groups given ethanol extract of *E. elatior* fruits 2.1 mg / 20 g BW, 4.2 / 20 g BW, 8.4 / 20 g BW starting from five days before LPS induction until seven days. Dose of ethanol extract *E. elatior* based on research by Fadiyah *et al*. (2018).
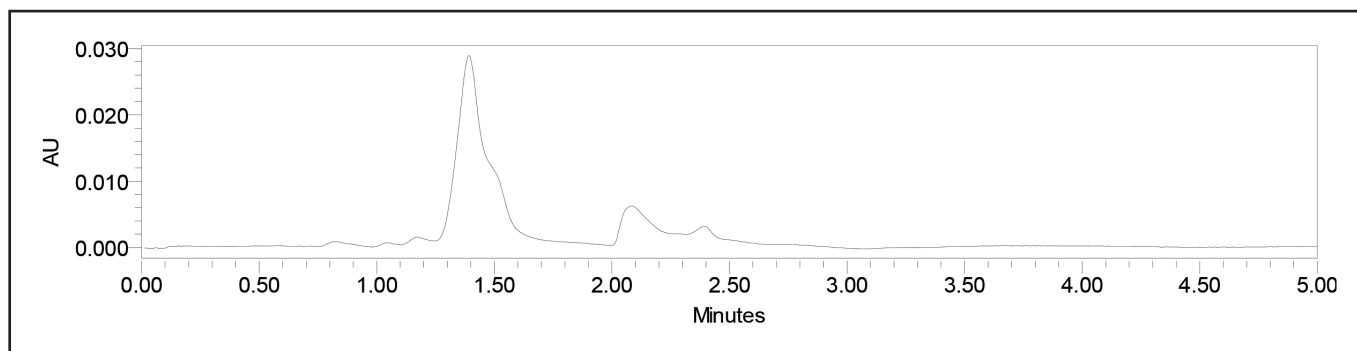
Figure 2. *Vanillic acid* standard chromatogram (RT 1.323 minutes); C18 (150 x 4.6 mm. I.D; 5μm), Mobile phase: Methanol: Water (70:30) isocratic, 1 mL/min, and λ 220 nm.

Measurement of NADPH oxidase activity
The NADPH oxidase activity were measured using the ELISA method. The tissue sample (10 mg) was homogeneously prepared with 220 µl NADPH oxidase buffer assay and placed on ice with a dose of tissue homogenizer. To eliminate cell debris and save the supernatant, centrifuge the lysates 10,000 times for 10 minutes at 40 °C. 1-50 µl of supernatant sample should be added to each of the 96 wells of a flat-bottomed transparent disk. With a 50 µl NADH oxidase buffer experiment, thoroughly mix all samples. As a control, prepare one well and add 50 µl of NADPH oxidase buffer assay. The plate was read at a wave length of 600nm at room temperature. The incubation time is determined by the sample's NADPH oxidase activity. It is recommended to measure the absorption process in the kinetic model and choose two time benchmarks (T1 and T2) in the linear range to measure NADPH activity. The standard NADPH oxidase curve can be read at the endpoint (at the end of 30 minutes of incubation). To calculate the NADPH oxidase activity in the sample, use the following equation:

Activity NADPH oxidase in the sample = $\frac{B}{\Delta T \ X \ P}$ = pmol/min/mg = µU/mg
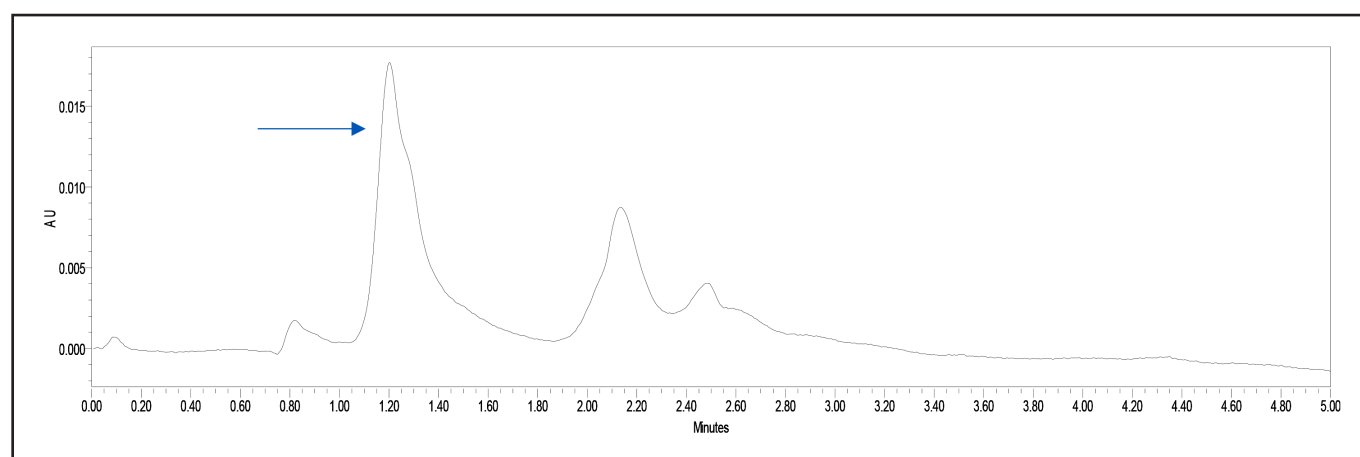
B : the amount of results from the NADPH oxidase standard curve
ΔT : the difference between T2 and T1 (minutes)
P : the amount of protein in the sample (mg)

Statistical analysis
The data were analyzed using Statistical Product and Service Solution (SPSS) 22.0 version for Windows. All collected data were presented in mean ± standard deviation. Before running the statistical analysis, the normality and homogeneity of the NADPH oxidase activity data were analyzed using Shapiro-Willk and Levene's test. One Way ANOVA was carried out to find out the difference of NADPH oxidase activity, followed by the Post-hoc Least Significant Difference (LSD) test. The results are considered significant if the p-value<0.05.

RESULTS

Based on the results of the high performance liquid chromatography test, each gram of the ethanol extract produced from the fruits of *E. elatior* contained 255 mg of VA (Figure 3).

This study used an IC_{50} (inhibitory concentration 50 %) test to measure the quantitative antioxidant activity. The outcomes demonstrated that VA had an IC_{50} value of 8.996 ppm while *E. elatior* fruit extract had an IC_{50} value of 5.079 ppm, indicating a higher level antioxidant activity.

Activity of NADPH oxidase in sepsis model
Differences in the activity of NADPH in the normal mice (N-1), N-2 group (negative control LPS only), N-3 group (the ethanol extract of *E. elatior* fruits of 2.1 mg / 20 gr), N-4 group (the ethanol extract of *E. elatior* fruits of 4.2 mg / 20 g) and N-5 group (the ethanol extract of *E. elatior* fruits of 8.4 mg / 20 g) were identified using the ANOVA test because the data in the study were normally distributed. Table 1 below shows the findings of the variations in NADPH activity in the N-1 group, N-2 group, N-3 group, N-4 group and N-5 group.

Table 1. Effects of the ethanol extract of *E. elatior* fruit on NADPH oxidase activity in various treatment

Treatment	NADPH (µl/mg)	p-*value*
N-1	6.867±0.217	<0,001**
N-2	18.651±0.163	<0,001**
N-3	10.153±0.450	<0,001**
N-4	8.909±0.100	<0,001**
N-5	7.862±0.357	<0,001**

* Significant p < 0.05.
** Significant p < 0,001.

Based on the results of the ANOVA assay, it was found that the various dosages of ethanol extract of *E. elatior* fruits had significant effects on NADPH oxidase activity, with p-value <0.05. The average value of NADPH oxidase activity in the N-1 group, N-2 group, N-3 group, N-4 group and N-5 group are 6.867±0.217, 18.651±0.163, 10.153±0.450, 8.909±0.100, and 7.862±0.357 respectively. Further test was carried out using the LSD post hoc test with the following result.

Figure 3. Ethanol extract of *E. elatior* fruits chromatogram. (RT = 1.223 minutes); C18 (150 x 4.6 mm. I.D; 5µm), Mobile phase: Methanol: Water (70:30) isocratic, 1 mL/min, and λ 220 nm.

Table 2. Post hoc test of NADPH oxidase activity in various treatment

| Comparisons	Mean Difference	p-*value*
N-1 vs N-2	-11.78338	<0,001**
N-3 vs N-1	3.285775	<0,001**
N-3 vs N-2	-8.49763	<0,001**
N-4 vs N-1	2.04200	<0,001**
N-4 vs N-2	-9.74138	<0,001**
N-4 vs N-3	-1.24375	<0,001**
N-5 vs N-1	0.99500	<0,001**
N-5 vs N-2	-10.78838	<0,001**
N-5 vs N-3	-2.29075	<0,001**
N-5 vs N-4	-1.04700	<0,001**

* Significant $p < 0.05$.
** Significant $p < 0,001$.

Table 2 shows that there was a significant difference in NADPH oxidase activity in the N-1 group, N-2 group ($p<0.001$), N-3 group ($p<0.001$), N-4 group ($p<0.001$) and N-5 group ($p<0.001$). According to Table 1 the N-3 group, N-4 group and N-5 group are known to effectively lower NADPH oxidase activity. Compared to the N-3 group and N-4 group, the N-5 group showed the lowest activity of NADPH oxidase. The administration of ethanol extract of *E. elatior* at 8.4 mg / 20 gr BW was the most effective in reducing NADPH oxidase activity.

DISCUSSION

This study aims to prove the hypothesis that the ethanol extract of *E. elatior* fruit can inhibit NADPH oxidase. Sepsis is currently defined as an uncontrolled host response to infection (Lopes *et al.*, 2022). Oxidative stress and inflammation are central mechanisms that play a key role in the pathophysiology of sepsis (Abelli *et al.*, 2022). Sepsis causes systemic infection, which results in a proinflammatory response and increase ROS generation (Lopes *et al.*, 2022). ROS are produced through several activities, one of which is the enzyme NADPH oxidase activity (Nguyen *et al.*, 2017). NADPH oxidase is a multicomponent enzyme that becomes active after the assembly of four cytosolic proteins (p47phox, p67phox, p40phox and Rac2) with transmembrane proteins (p22phox and gp91phox which form cytochrome b558) (Belambri *et al.*, 2018). NADPH oxidase acts as a catalyst in the formation of superoxide anion radicals and produces ROS, which cause oxidative stress and macromolecular damage. NADPH oxidase has seven known isoforms: NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2. This isoform is composed of various core catalytic subunits: p22-phox, p47-phox, p67-phox, p40-phox, activator DUOX 1, activator DUOX, NOXA1 and NOXO1 (Joshi & Khan, 2019).

In sepsis, there is an increase activity of the NADPH oxidase which synthesizes ROS in blood vessels (Wu *et al.*, 2007). NADPH oxidase is the main source of intracellular ROS generation in macrophages and neutrophils (Kong *et al.*, 2010). ROS generated by NADPH oxidase play an important role in antimicrobial defense and inflammation. Unregulated ROS release can lead to excessive pathology and inflammation (Nguyen *et al.*, 2017). To prevent the generation of ROS, NADPH oxidase activation needs to be strictly controlled (Belambri *et al.*, 2018). For more than 20 years, researchers have been looking for particular inhibitor for specific NADPH isozymes. NADPH oxidase activity has high therapeutic potential for several diseases including chronic kidney disease (CKD), pancreatic cancer, hypertension, cystic fibrosis, Parkinson's disease, pulmonary fibrosis, acute lung injury, heart failure, and ischemia (Joshi & Khan, 2019).
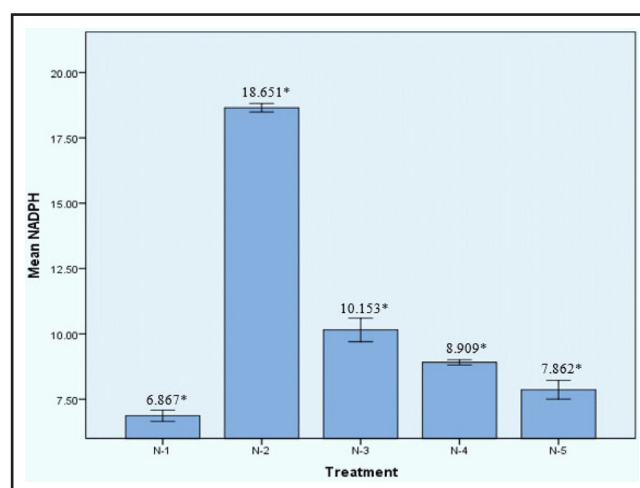
Figure 4. Description of NADPH oxidase activity in each treatment. The ethanol extract of *E. elatior* fruits decreased NADPH oxidase activity in experimental groups. Data represent the mean of five independent experiments and the best results in the group (N-5) treated with the ethanol extract of *E. elatior* 8.4 mg / 20 gr BW (N-5 mean: 7.862±0.357 $p < 0.001$).

Production of damage associated molecular patterns (DAMPs) and pathogens associated molecular patterns (PAMPs) induce the release of ROS by various cells such as endothelial cells, platelets and neutrophils. Continuously increasing ROS production will have negative impact on the patient's clinical conditions (Lopes *et al.*, 2022). ROS act as signaling molecules involved in vascular remodeling processes by modulating vascular tone and vascular structural changes leading to organ and mitochondrial injury (Savoia *et al.*, 2011). ROS can cause damage to proteins, lipids and cellular DNA, and disrupt mitochondrial function (Schieber & Chandel, 2014). ROS will also enhance adhesion proteins expression and promote iNos which can impair microvascular function in sepsis (Nguyen *et al.*, 2017) The NADPH oxidase complex in the cells will activate if there is stimulation of proinflammatory mediators, microbes, phagocytosis and activation of Pattern recognition receptors (PRRs) (Nguyen *et al.*, 2017). The formation of NADPH oxidase is initiated by activation of Rac2 through exchange of guanosine diphosphate to guanosine triphosphate and phosphorylation of p47-phox. The p47-phox subunit is a key regulator that causes the activating subunit Rac2 to translocate. Phospholarization of p47-phox is important because it allows the src-homologous domain to be opened (Briones *et al.*, 2012).

Based on Figure 4, the activity of NADPH oxidase test in this study showed that administration of ethanol extract of *E. elatior* fruits decreased NADPH oxidase with a p value <0.001. The most effective reduction in NADPH oxidase activity was achieved with an ethanol extract of *E. elatior* fruits dose of 8.4 mg /20 g compared to doses of 4.2 mg /20 g and 2.1 mg /20 g (Figure 4). The decrease in NADPH oxidase activity is most likely due to NADPH oxidase inhibition. According to a biocomputational study, VA has the potential to act as a NADPH oxidase inhibitor via p47-phox. VA has an average bond energy with p47-phox of -6.2 kl/mol with binding sites of Asp [221], Leu [260], Asp [261], Arg [302], Arg [316] (Laksono, 2020). VA compounds will cause inhibition of NADPH oxidase and further decrease ROS production. Based on the HPLC test, it was found that every one gram of ethanol extract of *E. elatior* fruit contained 255 mg of VA. In this study, it is possible that the inhibition of NADPH oxidase activity occurred due to the VA compound contained in the ethanol extract of *E. elatior* fruit.

There is currently no research on extract of *E. elatior* fruit as a NADPH oxidase inhibitor via p47-phox in sepsis model. A study by Wu *et al.* (2007) reported that there was an increase in NADPH oxidase and expression of the p47-phox subunit enzyme in microvascular endothelial cells of rats induced sepsis with LPS. The sepsis model was treated with high doses of ascorbic acid (500 and 1000 μM) and it was found that there was a decrease in the expression of NADPH oxidase and the expression of the p47-phox subunit enzyme (Wu *et al.*, 2007).

Furthermore, the ethanol extract of *E. elatior* fruit contains micronutrient compounds such as Manganese (Mn), Zinc (Zn), Selenium (Se), Copper (Cu) which function as antioxidants (Prasetiyo & Nasronudin, 2015). Micronutrient supplementation has been shown in studies to lower mortality in sepsis within 28 days (Prasetiyo & Nasronudin, 2015). *E. elatior* also contains flavonoids which can act as antibacterial by inhibiting bacterial cell nucleic acid synthesis, damaging the cytoplasm walls and inhibiting bacterial cell metabolism (Xie *et al.*, 2015). A study by Syafriana *et al.* (2021) showed that the extract of *E. elatior* inhibited the growth of *Staphylococcus epidermidis* at a concentration of 4% through cell wall destruction. In the present study, the reduce of NADPH oxidase activity in sepsis model treated with ethanol extract of *E. elatior* was due to NADPH oxidase inhibition and antibacterial activity. The ethanol extract of *E. elatior* fruits contains VA which can inhibit NADPH oxidase via 47 pox based in biocomputational study. This study has limitation including the variable parameters confounding factors. It is required to analyzed the phytochemical content of *E. elatior* fruit extract or purify the VA component to ensure that other secondary metabolites do not reduce NADPH oxidase activity.

ACKNOWLEDGEMENTS

Conflict of Interest
The authors declare that they have no conflict of interests.

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