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The ethyl acetate fraction of *Moringa oleifera* leaves effects on endothelial stress in rat sepsis model

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ABSTRACT

Background: Sepsis is a life-threatening organ dysfunction caused by the dysregulation of the host response to infection, facilitated by inflammation and endothelial stress. *Moringa oleifera* leaves fractioned with ethyl acetate (MO-EA fraction) is proposed to have the ability to suppress inflammation and oxidative stress through the control of NF-kB. This study aims to investigate the effect of the MO-EA fraction in a rat sepsis model.

Material and Methods: The study is a laboratory experimental study. The research was conducted on a total of 30 rats, equally divided into five groups. One group did not receive lipopolysaccharide (LPS) induction as negative control group, but the others received LPS induction and received variable doses of MO-EA fraction (0, 10, 20, and 40 mg/kg of bodyweight). The measured outcome were serum concentration of of heparanase, CRP, malondialdehyde (MDA), and immunohistologic expression of e-selectin, NF-kB in cells, and histopathological necrosis in the aorta and kidney.

Results: MO-EA fraction significantly decrease the serum levels of HPA, MDA on day 3 and 7, and lower the CRP serum level on day 7 ($p<0.05$). Other variables of interest did not show significant differences.

Conclusion: The administration of MO-EA fraction of any dose significantly lower the serum level of HPA and MDA in mice sepsis models on day 3 and 7, and lower CRP on day 3 but not on day 7. However, examnationa of NF-kB and e-selectin expressions, and necrosis in kidney proximal tubule and aortic endothelial cells did not show the benefit of MO-EA fraction in preventing cell damage.

Keywords: *Moringa oleifera* ethyl acetate fraction, Sepsis, Heparanase, Endothelial Stress

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INTRODUCTION

Complications of sepsis remained to be a major health problem in developed and developing countries, despite the advancement of medicine in terms of modernised intensive care units and highly trained doctors.[1] The dynamic and complex mechanism involving cell activation, neuroendocrine, coagulation, and fibrinolytic systems during infection contributes to the complex management of sepsis. These mechanisms produce sepsis markers that are important for sepsis evaluation.[2]

Serum heparanase (HPA), an enzyme that cuts and dissolves sulfate-containing fragments of proteoglycans from the glycocalyx layer of endothelial cells, acts as a marker of the glycocalyx degradation which can be used as a diagnostic tool for endothelial blood vessels dysfunction. In macrophages' cytoplasm, Nuclear factor-kappa B (NF-ƙB) in sepsis condition will bind with IƙB. The inflammation process and cytokines in sepsis also result in the expression of e-selectin, a selectin cell adhesion molecule on endothelial cells. E-selectin plays a vital role in recruiting leukocytes to the site of injury. During the early inflammation process, the increase in the serum CRP level can be detected early using hs-CRP.[3]

Moreover, the inflammation process produces oxidative stress which is measured using MDA as the marker. The oxidative stress eventually will lead to multiple organ dysfunction, marked by the activation of enzyme HPA and the histopathological changes in the aorta and the endothelium of proximal renal tubules. Furthermore, recent studies have shown that the glycocalyx, heparanase, sidecan-1, heparan sulfate, endocan and angiopoietin served as a septic marker in microvascular endothelial damage.[4]

The treatment of sepsis has been focused on antimicrobial therapy in combination with anti-inflammatory drugs. The research showed that the small dose of steroid suppresses the expression of NF-kB and caspase-3 in the sepsis rat model.[2] The *Moringa oleifera* (MO-EA) is widely

used in Indonesia for traditional medicine or supplementation, suggesting that it could have anti-inflammatory potential. Therefore, the researchers were interested in assessing the MO-EA fraction's anti-inflammatory effect in a sepsis rat model.

MATERIALS AND METHODS

Our study was an experimental research to compare the effect of MO-EA fraction in the sepsis mice model. This study was approved by Dr. Moewardi General Hospital Health Research Ethics Committee (ethical clearance letter no. 1.273/XI/HREC/2019).

Table 1A. The level of HPA in each group on day 3 after LPS induction

Group	HPA (in pg/mL) 3 days after LPS induction (mean±SD)	p-value
T1	322.80 ± 225.22[a]	
T2	239.35 ± 145.28[a]	
T3	251.65 ± 228.79[a]	<0.05
PC	996.05 ± 502.99[b]	
NC	231.32 ± 140.80[a]	

T1: Treatment group 1, T2: Treatment group 2, T3: Treatment group 3, PC: Positive control group, NC: Negative control group

Notes: the statistically significant difference in mean±standard deviation according to LSD test is shown when the mean± standard deviation is followed by different letter. When mean± standard deviation was followed by the same letter, it means that there is no significant difference statistically.

Table 1B. The level of HPA in each group on day 7 after LPS induction

Group	HPA (in pg/mL) 7 days after LPS induction (mean±SD)	p-value
T1	202.67 ± 154.52[a]	
T2	165.42 ± 81.55[a]	
T3	199.82 ± 58.66[a]	<0.05
PC	1154.53 ± 413.77[b]	
NC	137.70 ± 119.23[a]	

Table 2A. The level of MDA in each group on day 3 after LPS induction

Group	MDA (in ng/mL) 3 days after LPS induction (mean±SD)	p-value
T1	81.73 ± 8.42[a]	
T2	77.97 ± 14.04[a]	
T3	81.00 ± 17.75[a]	<0.05
PC	148.53 ± 101.64[b]	
NC	75.25 ± 4.13[a]	

Table 2B. The level of MDA in each group on day 7 after LPS induction

Group	MDA (in ng/mL) 7 days after LPS induction (mean±SD)	p-value
T1	117.57 ± 3.82[a]	
T2	112.62 ± 3.49[a]	
T3	113.03 ± 4.70[a]	<0.05
PC	142.92 ± 14.99[b]	
NC	109.43 ± 1.41[a]	

A total of 30 Wistar white male rat three to four months old with bodyweight between 170 and 200 grams were obtained from the Faculty Center, Universitas Gajah Mada, Indonesia. During the study, the rats were fed with standard BR1 adjusted to the average weight of their body and their drinks were not limited (*ad libitum*). The rats were kept in the temperature of 32°C with 12 hours dark and light cycle. These rats are randomly allocated into three different treatment groups, and a positive and a negative control groups. Each group had equal number of rats.

The rats in the positive control (PC) and the treatment groups (T1, T2, T3) were injected intraperitoneally with 0.25 mg lipopolysaccharide (LPS) per kilogram bodyweight to induce sepsis at the beginning of the study. The rats in the negative control group (NC) is injected with normal saline with the same procedure as other groups and were not fed with MO-EA fraction.

The *Moringa oleifera* (MO-EA fraction) that had been processed at Gadjah Mada University (UGM), Inter University Center (PAU) was fed using feeding tubes to the rats in group T1, T2, T3 at a dose of 10, 20, 40 mg/kg bodyweight respectively for five days before LPS injection. The MO-EA fraction feeding was continued everyday until seven days after LPS injection.

The serum concentration of HPA, CRP, malondialdehyde (MDA) were measured using method *enzyme-linked immunosorbent assay* (ELISA) on the third and seventh day of the LPS induction. The rats were sacrificed on the seventh day of the LPS induction to evaluate the level of expression of e-selectin, NF-kB in cells. A histopathology examination using hematoxylin eosin (HE) staining for evaluating the necrosis of the aortic endothelial cell and the renal proximal tubules cells was also conducted using a microscope.

The expression of NF-kB and the expression of e-selectin will be examined using immunohistochemistry; levels of HPA, levels of hs-CRP, and the levels of MDA will be examined using ELISA.

The data were analyzed using SPSS version 22. The statistical analysis for numerical variables is conducted with ANOVA test if the data is normally distributed. Otherwise, the Mann Whitney test will be used for the non-parametric test. The post-hoc analysis will be conducted with Least Significant Difference (LSD) test. A p-value of less than 0.05 is considered significant. The number of rats with variable level of expression of NF-kB, e-selectin, and necrosis are examined once on the seventh day of observation group.

Table 3A. The level of hs-CRP in each group on day 3 after LPS induction

Group	CRP (in ng/mL) 3 days after LPS induction (mean±SD)	p-value
T1	1.96 ± 0.41 [a]	
T2	1.82 ± 0.40 [a]	
T3	1.88 ± 0.31 [a]	<0.05
PC	3.31 ± 1.66 [b]	
NC	1.70 ± 0.16 [a]	

Table 3B. The level of hs-CRP in each group on day 7 after LPS induction

Group	CRP (in ng/mL) 7 days after LPS induction (mean±SD)	p-value
T1	0.68 ± 0.53	
T2	0.50 ±0.30	
T3	0.68 ±0.43	≥0.05
PC	1.15 ±0.44	
NC	0.35 ±0.19	

Table 4. The number of rats with variable NF-kB expressions in kidney and aorta

Group	NF-kB expressions						
	in kidney proximal tubule cells			in aortic endothelial cells			
	Low	Moderate	High	Negative	Low	Moderate	High
T1	1	5	0	1	2	2	1
T2	0	3	3	1	3	2	0
T3	4	2	0	1	0	0	5
PC	3	3	0	0	0	2	4
NC	1	2	3	3	2	1	0

Table 5. The number of rats with variable e-selectin expressions in kidney and aorta

Group	E-selectin expressions						
	in kidney proximal tubule cells			in aortic endothelial cells			
	Low	Moderate	High	Negative	Low	Moderate	High
T1	5	1	0	1	3	0	2
T2	6	0	0	2	2	2	0
T3	5	1	0	1	1	3	1
PC	2	2	2	0	0	2	4
NC	6	0	0	3	2	1	0

Table 6. The number of rats with variable level of necrosis in kidney and aorta

Group	Necrosis					
	kidney cells		aortic endothelial cells			
	No Necrosis	Weak necrosis	Negative	Low	Moderate	High
T1	2	4	0	3	3	0
T2	6	0	3	3	0	0
T3	2	4	1	4	1	0
PC	0	6	0	3	2	1
NC	6	0	5	0	1	0

RESULTS

The effect of MO-EA fraction to HPA and MDA serum levels

The concentration of HPA and MDA between the NC and PC shows a statistically significant difference in day 3 and 7. The concentration of HPA and MDA PC is significantly higher statistically when compared to T1, T2 and T3 ($p<0.05$) in day-3 and 7. But, in T1, T2, and T3, the differences are not statistically significant in day 3 and 7 (See Table 1A & Table 1B and Table 2A & Table 2B).

The effect of MO-EA fraction to CRP serum level

In day 3, the concentration of CRP between the NC and PC shows a statistically significant difference. The concentration of CRP in PC is significantly higher statistically when compared to T1, T2 and T3 ($p<0.05$) in day-3. But, in T1, T2, and T3, the differences are not statistically significant in day-3 (See Table 3A & B). There is no statistical difference in CRP level among the groups in day 7.

The effects of MO-EA fraction to the level of NF-kB, e-selectin expressions, and necrosis in kidney proximal tubule and aortic endothelial cells

The proportion of variable expressions of NF-kB, e-selectin, and necrosis in kidney proximal tubule and aortic endothelial cells among the groups are not showing any particular pattern (Table 4, 5, 6).

DISCUSSION

The study demonstrated that the administration of the MO-EA fraction in any dose lower HPA and MDA serum levels in rats on the third and seventh day after LPS induction. There is no similar study known to the researchers in evaluating the effect of MO-EA fraction to HPA in animal sepsis model. HPA usually increasis in glomerular filtration barrier abnormality. Some studies suggested that donregulation of NF-kB causes glycocalyx layer of glomerular filtration barrier disorder.[6,7] However, the expression of NF-kB in this study did not seem to be affected by the LPS induction nor the MO-EA fraction administration. Some in vitro studies claimed that MO-EA fraction significantly decreases the expression of NF-kB in macrophages.[7,8] Research had shown that the damage to kidneys in sepsis rat models are quite similar to the kidney damage observed in humans.[9] But, based on the inconcistency of the results shown by several studies, MO-EA fraction administration might not lower NF-kB in human.[9]

A study investigating e-selectin in sepsis and

non-sepsis patiet showed that e-selectin is expressed more in sepsis.[10] The administration of MO-EA fraction should lower the e-selectin expression if it has antiinflammatory effect. However, our study did not proof that this is the case. CRP is usually increased in sepsis.[11] The CRP level in the LPS induced rats was significantly higher than the negative control. Therefore, we successfully created a condition similar to sepsis. The CRP levels in rats fed with MO-AE fraction in the third and seventh day after the induction are significantly lowered than positive control group. This finding needs a further investigation whether it is applicable in human.

CONCLUSION

The administration of MO-EA fraction of any dose significantly lower the serum level of HPA and MDA in mice sepsis models on day 3 and 7, and lower CRP on day 3 but not on day 7. However, examination of NF-kB and e-selectin expressions, and necrosis in kidney proximal tubule and aortic endothelial cells did not show the benefit of MO-EA fraction in preventing cell damage.

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The authors self-funded the research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest in conducting and reporting the research.

AUTHORS CONTRIBUTION

The authors contributed equally in conducting the research and writing the manuscript.

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