Phaleria Macrocarpa's Extract Inhibits Autophagy Probably Through TNF-α in HUVEC Cell Culture

Leo Simanjuntak,[1,2] M Fidel Ganis Siregar,[3] Johannes C Mose,[4] Sarma N. Lumbanraja[3]
[1] Doctoral Program, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia
[2] Department of Obstetrics and Gynecology, Faculty of Medicine, Nommensen HKBP University, Medan, Indonesia
[3] Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Sumatera Utara, Medan, Indonesia
[4] Department of Obstetrics and Gynecology, Faculty of Medicine, Universitas Padjadjaran, Bandung, Indonesia
Korespondensi: Leo Simanjuntak, Email : leosimanjuntak66@gmail.com

Abstract
Objective: This study aimed to determine the effects of Phaleria macrocarpa's extract on TNF-α and LC3-II level and their correlation in preeclampsia-induced HUVEC.
Methods: This study used HUVEC culture as an in vitro model and Phaleria macrocarpa's extract, widely used as an anti-inflammation and antioxidant.
Results: Phaleria macrocarpa's extract reduce TNF-α level siginificantly at concentration of 7.813µg/mL and at 62.5µg/mL reduce TNF-α level to normal level. There was no significant decrease and reduction to normal level in LC3-II. TNF-α has a strong positive correlation with LC3-II (r=0.958), that reduced TNF-α level will decrease LC3-II levels, where a decrease in TNF-α level of 1pg/mL will reduce LC3-II levels by 0.413pg/mL.
Conclusion: Thus, Phaleria macrocarpa's extract might be used to overcome endothelial dysfunction and autophagy in preeclampsia.

Key words : Phaleria Macrocarpa, Preeclampsia, HUVEC, TNF-α, LC3-II

Ekstrak *Phaleria macrocarpa* Menghambat Otofagi Melalui TNF-α Pada Kultur Sel HUVEC

Abstrak:
Tujuan: Penelitian ini bertujuan untuk mengetahui efek ekstrak *Phaleria macrocarpa* pada inflamasi dan otofagi pada sel endotel dengan mengukur kadar TNF-α dan LC3-II pada HUVEC yang diinduksi serum preeklampsia.
Metode: Penelitian ini menggunakan kultur HUVEC sebagai model in-vitro yang banyak digunakan untuk memelajari patogenesis preeklampsia. *Phaleria macrocarpa* (Scheff.) Boerl juga dikenal sebagai Mahkota Dewa secara luas digunakan sebagai anti-inflamasi dan antioksidan
Hasil: Hasil menunjukkan ekstrak *Phaleria macrocarpa* menurunkan kadar TNF-α secara signifikan pada konsentrasi 7.813 µg/mL dan konsentrasi 62.5 µg/mL menurunkan kadar TNF-α ke kadar normal. Tidak terdapat penurunan signifikan rerata kadar LC3-II antara kontrol dan model PE dan dibutuhkan ekstrak *Phaleria macrocarpa* pada konsentrasi lebih dari 250 µg/mL untuk menurunkan kadar LC3-II pada model preeklamsia ke kadar hamil normal. Kadar TNF-α memiliki korelasi positif yang bermakna dengan LC3-II dengan tingkat korelasi sangat kuat (r = 0.847), dimana penurunan kadar TNF-α sebesar 1 pg/mL akan menurunkan kadar LC3-II sebesar 0.413 pg/mL.
Kesimpulan: Dengan demikian, ekstrak *Phaleria macrocarpa* dapat digunakan untuk mengatasi disfungsi endotel dan otofagi pada preeklampsia.

Kata kunci: Phaleria Macrocarpa, Preeklampsia, HUVEC, TNF-α, LC3-II

Introduction

Preeclampsia is one of the leading causes of maternal morbidity and mortality worldwide. It is estimated that maternal deaths worldwide are around 500,000 annually and about 10%−15% are due to preeclampsia and eclampsia.[1] In 2006 WHO reported that 16% of maternal deaths in developed countries due to hypertension in pregnancy, higher than due to bleeding of 13%, abortion of 8% and sepsis of 2%.[2]

Although there have been many studies but the etiopathogenesis of preeclampsia is still not fully elucidated but it is believed to be a multifactors. Thus preeclampsia is called the 'disease of theories'.[3]

Endothelial dysfunction plays an important role in the pathophysiology of preeclampsia. Under normal circumstances, endothelial cells maintain vascular integrity, regulating blood pressure, preventing intravascular coagulation, and regulating vascular smooth muscle tone by producing various substances including nitric oxide (NO), endothelin, prostacyclin and thromboxane.[4] Endothelial dysfunction occurs due to cytotoxic factors in the circulation such as superoxide anions (O_2) and H_2O_2, trophoblast debris, pro-inflammatory cytokines, metabolic factors, and anti-angiogenic factors produced by oxidative stressed placenta and causing excessive maternal inflammatory responses.[5]

Tumor Necrosis Factor-α (TNF-α) is considered as one of the potentially specific markers for preeclampsia. In endothelial cells TNF-α causes endothelial dysfunction by increasing oxidation of low-density lipoprotein (LDL), inhibiting eNOS enzymes causing NO levels decrease and increasing free radical production by xanthine oxidase enzyme, then binds to endothelial cells and produces an O_2^- anion in endothelial cells.[6-7]

TNF-α levels in preeclampsia placenta were significantly higher than normal pregnancies with ELISA method and allegedly TNF-α increases oxidative stress by stimulating the formation of ROS.[3]

Udenze et al.[8] found that TNF-α level in preeclampsia serum is significantly higher than in the serum of normal pregnancies (44.80 pg/mL vs 8.15 pg/mL).

Autophagy plays an important role in the pathophysiology of preeclampsia as observed in the failure of trophoblast invasion and spiral artery remodeling. Autophagy acts to increase extravillous trophoblast invasion and increase the remodeling of the spiral arteries under normal physiological hypoxia occurring early in pregnancy.[9-10] Autophagy can be induced by many overlapping factors such as nutritional deficiencies, growth factors deficiencies, and intracellular stress due to hypoxia.[11-13]

Autophagy-related proteins (ATG) such as Beclin-1 and LC3-II are some of the autophagy markers.[12-14] Strong evidence that autophagy has occurred is the formation of vesicles in the cytoplasm called autophagosomes. LC3-II is indispensable in the final stages of autophagosome formation so that it is used as a typical marker of autophagosome formation.[14-15] Oh et al.[11] found that the administration of TNF-α in trophoblast cell line cultures causes an increase in expression of LC3-II. TNF-α is thought to stimulate autophagy through mechanisms that depend on ROS.[16] thus it is believed that the autophagy process will be inhibited if oxidative stress and the inflammatory process are inhibited.

Preeclampsia treatment will only be successful and rational if based on understanding the disease pathophysiology. In an attempt to determine the pathophysiology of a disease, in vitro model research is considered the best and most effective way.[17]

HUVEC (Human Umbilical Vein Endothelial Cell) cell line culture and trophoblast cell line is an in vitro model

widely used to study the pathogenesis of preeclampsia.

Herbs or medicinal plants have been used traditionally as alternative medicine since ancient times. *Phaleria macrocarpa* (Scheff.) Boerl also known as Mahkota dewa belongs to the *Thymelaceae* family, that originated from Papua province, is very popular in Indonesia used in the treatment of various diseases such as cancer, hemorrhoids, diabetes mellitus, hypertension, and others. [18-20] The phenol and flavonoid compounds in the extract of *Phaleria macrocarpa* have high antioxidant and anti-inflammatory activity. [20-21]

The aim of this study is to determine the effects of *Phaleria macrocarpa* (Scheff.) Boerl Extract on Tumor Necrosis Factor – Alpha (TNF- α) and LC3-II Level In preeclampsia-induced Human Umbilical Vein Endothelial Cell (HUVEC).

Method

Serum samples used were obtained from women at >20−42 weeks of gestational age, which were diagnosed preeclampsia at Dr. Hasan Sadikin General Hospital. Research subjects have fulfilled inclusion and exclusion criteria.

HUVEC cell line ATCC CRL-1730 obtained from American Type Collection Culture. HUVEC cell line was growth into tissue culture flask (25 cm^2) containing RPMI 1640 media, 20% (v/v) FBS qualified (fetal bovine serum) supplementation, 10% endothelial supplement, 1% Penicillin G - Streptomycin solution stabilized, and 1% antimycotic Fungizone Amphotericin B and 1% gentamicin. The cells were then incubated at 37^{O}C and 5% CO_2 (v/v). Culture medium is replaced every 2−3 days. Then cells are passaged every seven days until reach 80-90% confluence.

Phaleria macrocarpa (Scheff.) Boerl was obtained from the Research Institute for Industrial Plants at Manoko, Lembang, West Java, Indonesia. The plant species was identified by the laboratory of Plant Taxonomy staff at Herbarium Bogoriense, Bogor, Indonesia.

As many as $6x10^5$ cells/mL induced with normal and preeclampsia serum, were placed into 60-well plate, then incubated at 37^{O}C and 5% CO_2 (v/v). Each well then was washed with 37^{O}C PBS 3−4 times. Furthermore, various concentrations of Phaleria macrocarpa's extract (0,977; 1,953; 3,906; 7,813; 15,625; 31,25; 62,5; 125; and 250 μg/mL) were added into each well, then incubated for 24 and 72 hours 37^{O}C and 5% CO_2 (v/v). Each well then was washed with 37^{O}C once for five minutes. Transfer the cells into centrifugation tube using 1.5 mL pipette. Centrifuged at 1.500 rpm for 10 minutes at 4°C. Use the supernatant as a sample for the ELISA method measurement of TNF-α and LC3-II levels, then the rest of the sample can be stored at -80 °C.

Data was analyzed with repeated ANOVA (analysis of variance) test which is followed by Bonferroni test as post hoc comparison test and Pearson correlation test and Linear regression to find correlation between the two variables.

Results

TNF-α levels in preeclampsia HUVEC culture model is higher than normal pregnancy HUVEC culture model. The TNF-α levels at 72 hours incubation time was lower than the 24 hours incubation time in both normal and preeclampsia models. LC3-II levels in preeclampsia HUVEC culture model is higher than normal pregnancy HUVEC culture model. The LC3-II levels at 72 hours incubation time was higher than the 24 hours incubation time in both normal and preeclampsia models.

Table 1 TNF-α levels (pg/mL) in preeclampsia and normal serum-induced HUVEC culture model treated with *Phaleria macrocarpa*'s extract in various concentrations incubated for 24 and 72 hours.

Phaleria macrocarpa's extract concentration (µg/mL)	24 H INCUBATION TIME		72 H INCUBATION TIME	
	NP* (Mean ± SD)	PE (Mean ± SD)	NP* (Mean ± SD)	PE (Mean ± SD)
Control	8.7 ± 0.04	18.7 ± 0.00	7.8 ± 0.03	17.8 ± 0.03
0.977	8.2 ± 0.00	18.3 ± 0.05	7.4 ± 0.01	17.4 ± 0.00
1.953	7.9 ± 0.00	17.9 ± 0.00	6.9 ± 0.00	16.7 ± 0.01
3.906	7.4 ± 0.07	15.3 ± 0.08	6.3 ± 0.02	14.6 ± 0.31
7.813	6.8 ± 0.00	14.5 ± 0.00	5.9 ± 0.05	13.7 ± 0.00
15.625	6.0 ± 0.00	12.7 ± 0.00	5.1 ± 0.00	11.4 ± 0.70
31.25	6.0 ± 0.00	10.1 ± 0.00	5.0 ± 0.01	9.3 ± 0.00
62.5	5.9 ± 0.01	8.5 ± 0.00	5.0 ± 0.00	7.6 ± 0.00
125	5.4 ± 0.01	7.6 ± 0.00	4.6 ± 0.00	6.8 ± 0.00
250	5.0 ± 0.00	6.1 ± 0.00	4.1 ± 0.00	5.4 ± 0.00

NP : Normal Pregnancy

Table 2 TNF-α levels (pg/mL) mean comparison before and after various concentrations of *Phaleria macrocarpa*'s extract treatment at 24 hours and 72 hours incubation time in preeclampsia HUVEC culture model.

Phaleria macrocarpa's extract concentration (µg/mL)	24 H INCUBATION TIME		72 H INCUBATION TIME	
	PE (Mean ± SD)	P value*	PE (Mean ± SD)	P value*
Control	18.7 ± 0.00		17.8 ± 0.03	
0.977	18.3 ± 0.05	0.23	17.4 ± 0.00	1.00
1.953	17.9 ± 0.00	0.47	16.8 ± 0.01	0.36
3.906	15.3 ± 0.09	0.03	14.7 ± 0.31	1.00
7.813	14.5 ± 0.00	0.02	13.7 ± 0.00	0.18
15.625	12.8 ± 0.00	0.02	11.5 ± 0.71	1.00
31.25	10.1 ± 0.00	0.01	9.3 ± 0.00	0.08
62.5	8.6 ± 0.00	0.01	7.7 ± 0.00	0.07
125	7.6 ± 0.00	0.01	6.8 ± 0.00	0.07
250	6.1 ± 0.00	0.23	5.4 ± 0.00	0.06

* : statistically significant if $p < 0.05$

Table 3 LC3-II levels (pg/mL) in preeclampsia and normal serum-induced HUVEC culture model treated with *Phaleria macrocarpa*'s extract in various concentrations incubated for 24 and 72 hours.

Phaleria macrocarpa's extract concentration (µg/L)	24 H INCUBATION TIME		72 H INCUBATION TIME	
	NP* (Mean ± SD)	PE (Mean ± SD)	NP* (Mean ± SD)	PE (Mean ± SD)
Control	0.9 ± 0.00	6.5 ± 0.14	1.7 ± 0.07	6.3 ± 0.43
0.977	0.8 ± 0.02	6.3 ± 0.15	1.6 ± 0.02	6.0 ± 0.53
1.953	0.7 ± 0.00	6.0 ± 0.00	1.5 ± 0.00	6.3 ± 0.00
3.906	0.7 ± 0.00	5.9 ± 0.00	1.5 ± 0.00	5.9 ± 0.00
7.813	0.6 ± 0.00	5.8 ± 0.00	1.5 ± 0.00	5.9 ± 0.00
15.625	0.5 ± 0.01	5.4 ± 0.00	1.3 ± 0.00	5.5 ± 0.00
31.25	0.5 ± 0.00	3.9 ± 0.00	1.3 ± 0.00	4.0 ± 0.00
62.5	0.5 ± 0.00	2.3 ± 0.14	1.2 ± 0.01	2.4 ± 0.07
125	0.4 ± 0.00	2.1 ± 0.01	1.0 ± 0.01	2.1 ± 0.03
250	0.3 ± 0.00	1.2 ± 0.03	1.0 ± 0.00	1.3 ± 0.08

NP : Normal Pregnancy

Table 4 LC3-II levels (pg/mL) mean comparison before and after various concentrations of *Phaleria macrocarpa*'s extract treatment at 24 hours and 72 hours incubation time in preeclampsia HUVEC culture model

Phaleria macrocarpa's extract concentration (µg/mL)	24 H INCUBATION TIME		72 H INCUBATION TIME	
	PE (Mean ± SD)	P value*	PE (Mean ± SD)	P value*
Control	6.5 ± 0.14		6.3 ± 0.44	
0.977	6.3 ± 0.15	0.27	6.0 ± 0.53	1.00
1.953	6.0 ± 0.00	1.00	6.3 ± 0.00	1.00
3.906	5.9 ± 0.00	1.00	5.9 ± 0.00	1.00
7.813	5.8 ± 0.00	1.00	5.9 ± 0.00	1.00
15.625	5.4 ± 0.00	1.00	5.5 ± 0.00	1.00
31.25	3.9 ± 0.00	1.00	4.0 ± 0.00	1.00
62.5	2.3 ± 0.14	1.00	2.4 ± 0.07	1.00
125	2.1 ± 0.01	0.73	2.1 ± 0.03	1.00
250	1.2 ± 0.03	0.42	1.3 ± 0.08	1.00

* : statistically significant if $p < 0.05$

Table 1. shows TNF-α levels mean in difference preeclampsia and normal serum-induced HUVEC culture model treated with *Phaleria macrocarpa*'s extract in various concentrations incubated for 24 and 72 hours.

Table 2 shows TNF-α level decreased in preeclampsia serum-induced HUVEC ATCC CRL 1730 following increased *Phaleria macrocarpa*'s extract concentration. TNF-α level significantly decreased after exposure of *Phaleria macrocarpa*'s extract on concentration 7.813 µg/mL. ($p<0,05$).

Table 3. shows LC3-II levels mean difference in preeclampsia and normal serum-induced HUVEC culture model treated with *Phaleria macrocarpa*'s extract in various concentrations incubated for 24 and 72 hours.

Table 4 shows LC3-II level decreased in preeclampsia HUVEC model following increased *Phaleria macrocarpa*'s extract concentration. However, there was no significant decrease in mean LC3-II levels between control and PE model.

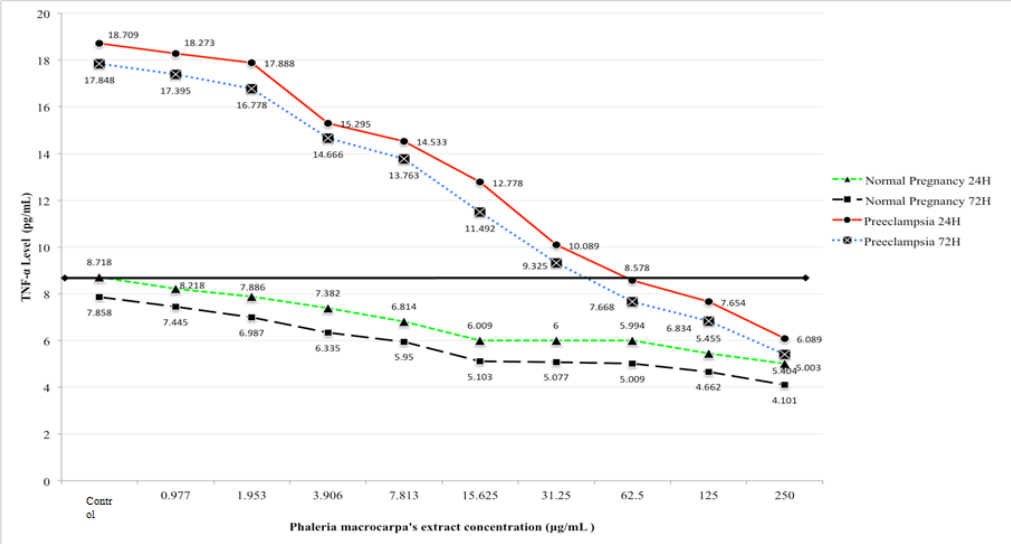

Figure 1. TNF-α levels in relation with Phaleria macrocarpa's extract concentration

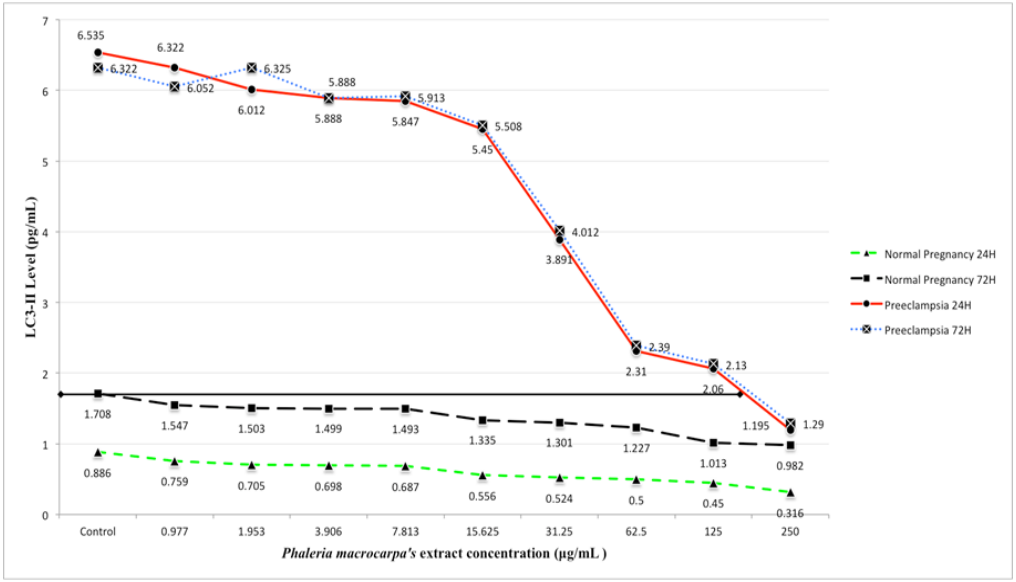

Figure 2. LC3-II levels in relation with Phaleria macrocarpa's extract concentration

Figure 1 shows that Phaleria macrocarpa's extract at concentration of 62.5 µg/mL reduce TNF-α level in preeclampsia model to normal pregnancy level.

Figure 2 shows that *Phaleria macrocarpa's* extract at concentration more than 250 µg/mL needed to reduce LC3-II level in preeclampsia model to normal pregnancy level.

Based on Pearson correlation test and Linear regression, TNF-α has a strong positive correlation with LC3-II (r = 0.958). The correlation between values indicates that when reduced TNF-α level, a proportional decrease in LC3-II levels also occurs, where a decrease in TNF-α level of 1 pg/mL will reduce LC3-II levels by 0.413 pg/mL.

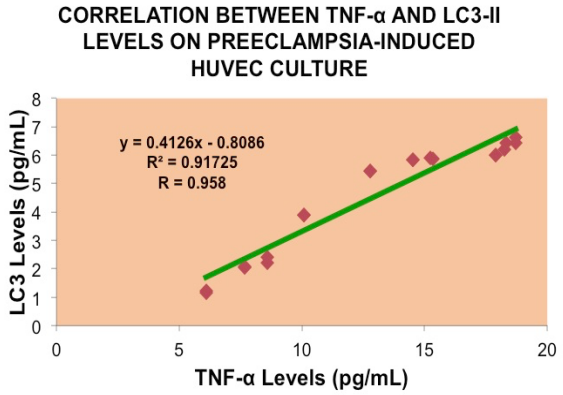

Figure 3 Graph showing correlation between TNF-α and LC3-II levels

Discussion

This were the first study to evaluate the effects of *Phaleria macrocarpa* (Scheff.) Boerl extract on Tumor Necrosis Factor – Alpha (TNF- α) and Protein Light Chain 3-II (LC3-II) level in Preeclampsia-Induced Human Umbilical Vein Endothelial Cell (HUVEC). Preeclampsia and eclampsia have been known since ancient times but their pathophysiology is still not clearly understood.

There is compelling evidence that endothelial dysfunction plays a role in the pathophysiology of preeclampsia. A consistent finding is the presence of glomerular endotheliosis in more than 70% of primiparous preeclampsia patients and this glomerular endotheliosis will disappear after delivery.

Oh et al.[11] found that the administration of TNF-α in trophoblast cell line cultures causes an increase in expression of LC3-II. TNF-α is thought to stimulate autophagy through mechanisms that depend on ROS [16] thus it is believed that the autophagy process will be inhibited if oxidative stress and the inflammatory process are inhibited.

To date, invitro research using HUVEC has been done a lot recently. Previous invitro research on HUVEC cultures by treating with anti-inflammatory and antioxidant compounds such as curcumin and Papua ant nest (*Myrmecodia pendens*) decrease oxidative stress and inflammation characterized by decreased levels of MDA, and TNF-α. These studies conclude that the Papuan ant nests and curcumin have a therapeutic effect on preeclampsia.[22-23]

TNF-α is considered as one of the potentially specific markers for preeclampsia and contributes to the formation of free radicals such as peroxides (H_2O_2), and superoxide (O_2).[6] Autophagy can be induced by many overlapping factors such as nutritional deficiencies, growth factors deficiencies, and intracellular stress due to hypoxia. LC3-II is used as a typical marker of autophagosome formation in autophagy.

In this study results showed TNF-α levels in preeclampsia HUVEC culture model was higher than normal pregnancy HUVEC culture model. TNF-α level decreased in preeclampsia and normal serum-induced HUVEC ATCC CRL 1730 culture following increased *Phaleria macrocarpa*'s extract concentration. TNF-α level significantly decreased after exposure of *Phaleria macrocarpa*'s extract on concentration 7.813 µg/mL. Phaleria macrocarpa's extract at concentration of 62.5 µg/mL reduce TNF-α

level to normal level.

This study shows LC3-II levels mean difference in preeclampsia and normal serum-induced HUVEC culture model treated with *Phaleria macrocarpa*'s extract in various concentrations incubated for 24 and 72 hours. LC3-II level decreased in preeclampsia and normal serum-induced HUVEC ATCC CRL 1730 culture following increased *Phaleria macrocarpa*'s extract concentration. However, there was no significant decrease in mean LC3-II levels between control and PE model treated with *Phaleria macrocarpa*'s extract in various concentrations incubated for 24 and 72 hours and *Phaleria macrocarpa's* extract at concentration more than 250 µg/mL needed to reduce LC3-II level in preeclampsia model to normal pregnancy level.

TNF-α has a strong positive correlation with LC3-II (r=0.958). The correlation between values indicates that when reduced TNF-α level, a proportional decrease in LC3-II levels also occurs, where a decrease in TNF-α level of 1 pg/mL will reduce LC3-II levels by 0.413 pg/mL.

Although there are many studies about *Phaleria macrocarpa*, one potential limitation in this study is the lack of prior research studies about the toxic profile or side effects of *Phaleria macrocarpa*'s extract specifically when used in an *in-vivo* model. We selected HUVEC culture, as endothelial dysfunction mainly happened in endothelial cells. This limitation encourages further clinical trials as the biologically active *Phaleria macrocarpa*'s extract compound can be further used for preeclampsia treatment and prevention.

The result of present study suggests that *Phaleria macrocarpa*'s extract contains anti-inflammatory activity proven by decreased level of TNF-α. It was also described that TNF-α level decreased in preeclampsia and normal serum-induced HUVEC ATCC CRL 1730 following increased *Phaleria macrocarpa*'s extract concentration. Thus, *Phaleria macrocarpa*'s extract might be used as agent to restore endothelial dysfunction in preeclampsia. Since the decreased the level of TNF-α in preeclampsia-induced HUVEC ATCC CRL 1730 culture, further clinical studies regarding the use of *Phaleria macrocarpa*'s extract in treatment are encouraged.

Conclusion

The *Phaleria macrocarpa*'s extract reduce TNF-α level siginificantly at concentration of 7.813 µg/mL in preeclampsia-induced HUVEC ATCC CRL 1730 culture. Phaleria macrocarpa's extract at concentration of 62.5 µg/mL reduce TNF-α level to normal level. There was no significant decrease in mean LC3-II levels between control and PE model treated with *Phaleria macrocarpa*'s extract in various concentrations incubated for 24 and 72 hours and *Phaleria macrocarpa's* extract at concentration more than 250 µg/mL needed to reduce LC3-II level in preeclampsia model to normal pregnancy level. TNF-α has a strong positive correlation with LC3-II, reduced TNF-α level will decrease LC3-II levels, where a decrease in TNF-α level of 1 pg/mL will reduce LC3-II levels by 0.413 pg/mL.

Conflict of Interest

The authors whose names are listed above certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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