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# The Effects of *Phaleria macrocarpa* (Scheff.) Boerl Extract on Tumor Necrosis Factor-Alpha (TNF-α) Level in Preeclampsia-Induced Human Umbilical Vein Endothelial Cell (HUVEC) Culture

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Abstract **:** Preeclampsia is a major cause in both maternal and perinatal mortality and morbidity. The etiopathogenesis of preeclampsia is still not fully elucidated but it is believed to be a multifactor. Endothelial dysfunction plays a big role in the pathophysiology of preeclampsia. Tumor Necrosis Factor-α (TNF-α) is considered as one of the potentially specific markers for preeclampsia. In vitro model research is considered the best and most effective way to understand the disease pathophysiology. HUVEC (Human Umbilical Vein Endothelial Cell) culture is an in vitro model widely used to study the pathogenesis of preeclampsia. *Phaleria macrocarpa* (Scheff.) Boerl also known as Mahkota Dewa is widely used as an anti-inflammation and antioxidant because of alkaloids, saponins, flavonoids and polyphenols properties. This study aimed to determine the effects of *Phaleria macrocarpa* Extract on Tumor Necrosis Factor – Alpha (TNF- α) Level In preeclampsia-induced Human Umbilical Vein Endothelial Cell (HUVEC). Our results showed the *Phaleria macrocarpa*'s extract reduce TNF-α level siginifcantly at concentration of 7.813 µg/mL. *Phaleria macrocarpa*'s extract at concentration of 62.5 µg/mL reduce TNF-α level to normal level. Thus, *Phaleria macrocarpa*'s extract might be used as agent to overcome endothelial dysfunction in preeclampsia.

Keywords **:** Phaleria macrocarpa, preeclampsia, HUVEC, TNF-α

## 1. 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 (Maynard et al. 2008). 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% (Cunningham et al. 2014).

Preeclampsia and eclampsia also adversely affect the fetus and the neonates. It is estimated that 15% of preterm births due to preeclampsia, where labor has to be performed to prevent the progression of preeclampsia (Roberts & Gammill 2005).

Although there have been many studies but the etiopathogenesis of preeclampsia is still not fully elucidated but it is believed to be a multifactors. Therefore preeclampsia remains a 'disease of theories'. The difficulties increase because the syndrome of preeclampsia usually occurs in the third trimester when the underlying disorder has occurred in the early stages of placentation, thus difficult to understand its progression (Cross 1996).

Endothelial dysfunction plays an important role in the pathophysiology of preeclampsia. Endothelial dysfunction is defined as an altered state of endothelial cell differentiation in response to sublethal injury or cytokine stimulation (Hubel 1999). 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 (Davidge 1998; Scalera , Schlembach & Beinder 2001). Rodgers et al. (1988) suggests that endothelial dysfunction occurs due to cytotoxic factors in the circulation. Impaired utero-placental perfusion in PE causes hypoxia, ischemia, placental oxidative stress, so the placenta produces free radicals such as superoxide anions ($O_2^-$) and $H_2O_2$, trophoblast debris, pro-inflammatory cytokines, and antiangiogenic factors which are thought to cause vascular endothelial dysfunction and causing excessive maternal inflammatory responses. Systemic maternal vascular endothelial dysfunction is the underlying cause of clinical manifestations in preeclampsia (Roberts et al. 1989).

Tumor Necrosis Factor-α (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 ($O_2^-$) (Amash et al. 2010). In endothelial cells TNF-α causes endothelial dysfunction by increasing oxidation of low-density lipoprotein (LDL) (Maziere, Auclair & Maizere 1994), inhibiting eNOS enzymes causing NO levels decrease (Zhang et al. 2009) and increasing free radical production by xanthine oxidase enzyme, then binds to endothelial cells and produces an $O_2^-$ anion in endothelial cells (Page et al., 1997).

Wang & Walsh (1996) found that TNF-α levels in placenta preeclampsia were significantly higher than normal pregnancies (17.32 ± 1.97 pg / mg protein vs 11.62 ± 0.93 pg / mg protein) with ELISA method and allegedly TNF-α increases oxidative stress by stimulating the formation of ROS. Hayashi et al. (2005) found that TNF-α level in preeclampsia serum is significantly higher than in the serum of normal pregnancies (4.68 pg/mL vs 3.31 pg/mL).

Zuspan (1978) suggested that PE 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 (Orendi et al. 2011). 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.

The preventions of preeclampsia consist of primary, secondary, and tertiary prevention. Primary prevention aims to prevent the onset of disease by avoiding pregnancy with contraception because the pathogenesis of preeclampsia remains unclear. Secondary prevention aims to inhibit the disease progression before the onset of clinical manifestations. Tertiary prevention aims to prevent the complications of a disease, in preeclampsia, the complications such as seizures, HELLP syndrome, and IUGR, which tertiary prevention can be interpreted as treatment Tertiary prevention includes regular antenatal examination, appropriate referral, anti-hypertensive administration, anti-convulsant administration, and appropriate timing of delivery (Dekker & Sibai 2001)..

The use of traditional medicines in Indonesia is part of a culture that has been going on since long time ago. Act No. 381 of 2007 on national traditional drug policy regulates the development of traditional medicines in order to obtain good quality, safe, and scientifically tested traditional medicine (Departemen Kesehatan RI 2007).

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, allergies, liver disease, heart disease, kidney disease, hypertension, migraine, skin diseases and others (Hendra et al. 2011; Anggraini & Lewandowsky 2015; Alara, Alara & Olalere 2016). *Phaleria macrocarpa* (Scheff.) Boerl is widely used as an antioxidant because of alkaloids, saponins, flavonoids and

polyphenols properties. The phenol and flavonoid compounds in the extract of *Phaleria macrocarpa* have antioxidant and anti-inflammatory activity (Tiwari 2001; Hendra et al. 2011).

To date there has been no research on the effect of the Phaleria macrocarpa's extract on the levels of TNF-α in preeclampsia-induced Human Umbilical Vein Endothelial Cell (HUVEC). The aim of this study is to determine the tffects of *Phaleria macrocarpa* (Scheff.) Boerl Extract on Tumor Necrosis Factor – Alpha (TNF- α) Level In preeclampsia-induced Human Umbilical Vein Endothelial Cell (HUVEC).

# 2. MATERIALS AND METHOD

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

## 2.1. Cell Culture

HUVEC cell line was obtained from American Type Collection Culture with ATCC CRL-1730 code number. 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 $37^OC$ 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.

## 2.2. Phaleria macrocarpa's Extract

*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.

## 2.3. Measurement of TNF- α Level

As many as $6x10^5$ cells/mL induced with normal and preeclampsia serum, were placed into 60-well plate, then incubated at $37^OC$ and 5% $CO_2$ (v/v). Each well then was washed with $37^OC$ 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 $37^OC$ and 5% $CO_2$ (v/v). Each well then was washed with $37^OC$ 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, then the rest of the sample can be stored at -80 °C.

## 2.4. Data Analysis

Data distribution was analyzed with Shapiro-Wilk normality test. Data was analyzed with repeated ANOVA (analysis of variance) test and followed by Bonferroni test as post hoc comparison test.

## 3. RESULTS

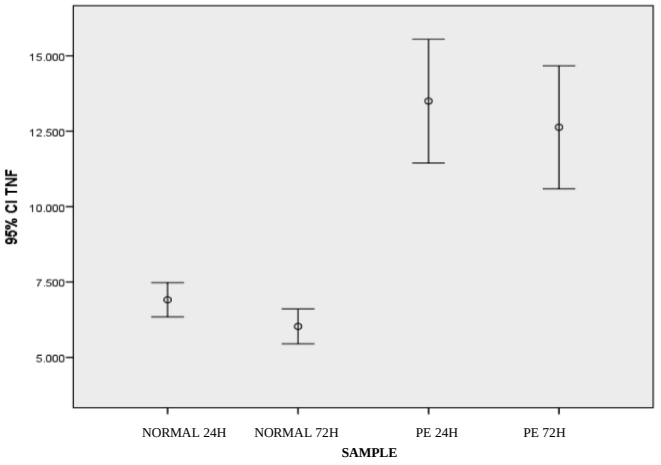


Figure 1. TNF-α levels in normal and preeclampsia-induced HUVEC based on incubation time.

As shown in figure 1 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.

Variables tested in this study were normally distributed both in normal and preeclampsia model treated with Phaleria macrocarpa's extract in various concentrations incubated for 24 and 72 hours.

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 | 8.718 ± 0.043 | 18.709 ± 0.007 | 7.858 ± 0.029 | 17.848 ± 0.035 |
| 0.977 | 8.218 ± 0.003 | 18.273 ± 0.051 | 7.445 ± 0.015 | 17.395 ± 0.007 |
| 1.953 | 7.886 ± 0.005 | 17.888 ± 0.001 | 6.987 ± 0.006 | 16.778 ± 0.015 |
| 3.906 | 7.382 ± 0.071 | 15.295 ± 0.087 | 6.335 ± 0.02 | 14.666 ± 0.312 |
| 7.813 | 6.814 ± 0.007 | 14.533 ± 0.000 | 5.950 ± 0.057 | 13.763 ± 0.000 |
| 15.625 | 6.009 ± 0.003 | 12.778 ± 0.000 | 5.103 ± 0.007 | 11.492 ± 0.708 |
| 31.25 | 6.000 ± 0.000 | 10.089 ± 0.000 | 5.077 ± 0.014 | 9.325 ± 0.000 |
| 62.5 | 5.994 ± 0.006 | 8.578 ± 0.000 | 5.009 ± 0.000 | 7.668 ± 0.001 |
| 125 | 5.455 ± 0.001 | 7.654 ± 0.000 | 4.662 ± 0.000 | 6.834 ± 0.001 |
| 250 | 5.003 ± 0.000 | 6.089 ± 0.000 | 4.101 ± 0.001 | 5.404 ± 0.000 |

NP : Normal Pregnancy

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. 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 | 18.709 ± 0.007 | | 17.848 ± 0.035 | |
| 0.977 | 18.273 ± 0.051 | 1.000 | 17.395 ± 0.007 | 1.000 |
| 1.953 | 17.888 ± 0.001 | 0.227 | 16.778 ± 0.015 | 0.362 |
| 3.906 | 15.295 ± 0.087 | 0.470 | 14.666 ± 0.312 | 1.000 |
| 7.813 | 14.533 ± 0.000 | 0.034 | 13.763 ± 0.000 | 0.175 |
| 15.625 | 12.778 ± 0.000 | 0.024 | 11.492 ± 0.708 | 1.000 |
| 31.25 | 10.089 ± 0.000 | 0.017 | 9.325 ± 0.000 | 0.082 |
| 62.5 | 8.578 ± 0.000 | 0.014 | 7.668 ± 0.001 | 0.070 |
| 125 | 7.654 ± 0.000 | 0.013 | 6.834 ± 0.001 | 0.065 |
| 250 | 6.089 ± 0.000 | 0.012 | 5.404 ± 0.000 | 0.058 |

* : statistically significant if $p < 0.05$

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. ($p<0,05$).

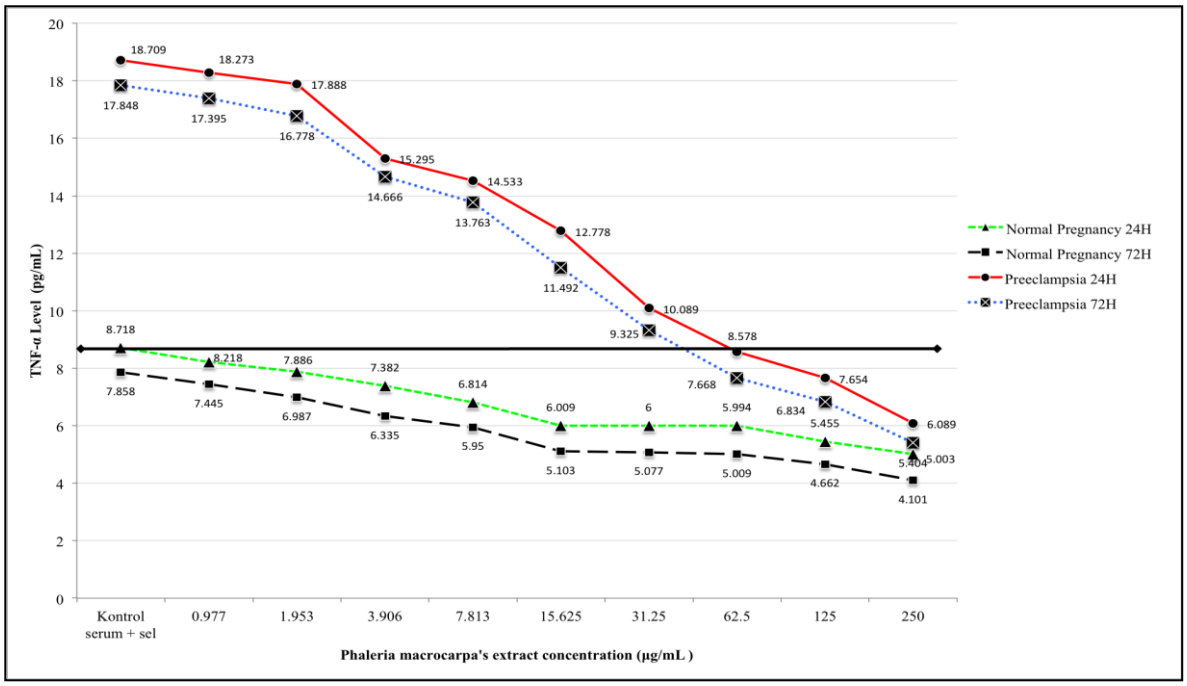


Figure 2. TNF-α levels in relation with Phaleria macrocarpa's extract concentration

Figure 2 shows that Phaleria macrocarpa's extract at concentration of 62.5 µg/mL reduce TNF-α level in preeclampsia model to normal pregnancy level.

## 4. DISCUSSION

This were the first study to evaluate the effects of *Phaleria macrocarpa* (Scheff.) Boerl extract on Tumor Necrosis Factor – Alpha (TNF- α) 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. Abnormal trophoblast invasion and placental perfusion disorders are thought to be the underlying cause of preeclampsia.

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 (Roberts et al., 1989).

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 (Yeni et al., 2017, Gunardi et al., 2016).

Tumor Necrosis Factor-α (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 ($O_2^-$) (Amash et al. 2010). The study by Yoshizumi et al. (1993) showed TNF-α decrease NOS mRNA levels in HUVEC, indicating a decrease in NO levels as vasodilators.

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.

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.


## 5. ACKNOWLEDGEMENT

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.


## 6. 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.

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