

Potential Of Mahkota Dewa Fruit (*Phaleria macrocarpa*) Flavonoids Extract and Dienogest to Reduce IL-17A Levels in Mice Endometriosis Model

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Abstract: Endometriosis is an inflammatory disease that occurs in glands outside the endometrium. Interleukin 17 (IL-17) is a proinflammatory cytokine degrading Th17 cells, which play an essential role in several inflammatory diseases. IL-17 expression is upregulated in serum, peritoneal fluid (PF), and endometriotic lesions from patients with endometriosis. Usually, treatment for endometriosis is dienogest. However, it has many side effects if used long-term. Fruit and seeds of *Phaleria macrocarpa* have various critical biological activities in the form of extracts such as antimicrobial, anti-inflammatory, and antioxidant activities. This study aims to analyze the effect of comparing flavonoid fruit extract of *Phaleria macrocarpa* and dienogest on IL-17A levels in endometriosis mice. A total of 35 endometriosis model mice were divided into seven groups, namely the group of healthy mice (without any treatment), the endometriosis group, and the endometriosis group, which were given the treatment of flavonoid fruit extracts of *Phaleria macrocarpa* and dienogest. The sample used in this study was peritoneal fluid. The enzyme-linked immunosorbent assay (ELISA) measured IL-17A levels in each observation group. The hypothesis was tested using One-Way ANOVA analysis with SPSS software version 26. This study found a significant difference ($p<0.05$) between the mean values of the positive control group and the negative control group. There was also a significant difference ($p<0.05$) in the mean value IL-17 between the treatment groups that were given *sphalerite macrocarpa* and dienogest extracts. Flavonoid fruit extract of *Phaleria macrocarpa* had a significant effect on reducing IL-17A levels in endometriosis model mice, and there is a significant difference with dienogest. Thus, the flavonoid fruit extract of *Phaleria macrocarpa* can potentially treat endometriosis by decreasing inflammatory factors.

Keywords: Dienogest; flavonoid; interleukin 17A; *Phaleria macrocarpa*.

INTRODUCTION

Endometriosis is an estrogen-dependent inflammatory disease defined as the presence of endometrial glands and stroma outside the uterine cavity, especially in the peritoneum of the pelvis and ovaries (Yuan et al., 2017). Endometriosis is a proliferative inflammatory chronic disease that affects 5-10% of women of reproductive

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age (Dietrich et al., 2023). Prevalence and incidence of endometriosis vary widely, leading to uncertainty about the true frequency of endometriosis or the validity of measured changes over time (Ghiasi et al., 2020).

The pathogenesis of endometriosis is still not known with certainty. Many theories and hypotheses try to explain the pathogenesis of endometriosis. Furthermore, until now, the most widely accepted theory to explain the etiology of endometriosis is the theory of retrograde menstruation. During menstruation, menstrual fragments originating from the eutopic endometrium travel back through the fallopian tubes into the peritoneal cavity, where these cells then implant into extra-uterine ectopic sites (Zhou et al., 2020). Interleukin 17 (IL-17) is a Th17 cell-derived proinflammatory cytokine that plays an essential role in several inflammatory diseases. IL-17 expression is upregulated in serum, peritoneal fluid (PF), and endometriotic lesions from patients with endometriosis. The IL-17 cytokine family contains six structurally related cytokines (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F) that share sequence homology and five corresponding receptors. IL-17A is the most characteristic first member (Shi et al., 2022).

Endometriosis therapy can be done with surgery or medication. One of the therapies currently used in endometriosis patients is the drug dienogest. Dienogest is a synthetic progesterone hormone drug used in the treatment of endometriosis. Dienogest works by suppressing estradiol production and preventing tissue growth. The most common side effects of dienogest are changes in menstrual patterns, breast pain, and liver and urinary disorders (Mehdizadeh Kashi et al., 2022).

Mahkota Dewa (*Phaleria macrocarpa*) is a herbal plant often used by the public as an alternative herbal medicine for endometriosis. *Phaleria macrocarpa* extract is reported to have been widely used for many pharmacological activities, including anti-tumor, anti-hyperglycemic, anti-inflammatory, anti-diarrheal, vasodilator, anti-oxidant, anti-viral, anti-bacterial and anti-fungal effects (Altaf et al., 2013). Phytochemicals in the mahkota dewa (*Phaleria macrocarpa*) include from fruit, seeds, Leaves, and fruit peels contain alkaloids, tannins, terpenoids, and flavonoids (Altaf et al., 2013). Besides that, the photo components in the fruit flavonoid extract of *Phaleria macrocarpa* contain various chemical elements, namely eriodictyol, glycerin, 5-O-methyl genistein, 4-(8-Isopropenyl-3, 4, 8, 9-tetrahydro-2H-furor [2,3-H] chrome-3yl)-1,3-benzenediol, 3,5-dihydroxy-2-(4-hydroxy phenyl) 4-oxo-3,4-dihydro-2H chromene heksopira Noida, (+)-catechin 7-O-beta-D-xyloside, (-)-8-prenylnaringenin, (\pm)-naringenin, Apigenin trimethyl ester (Maharani et al., 2021). Flavonoids weaken proinflammatory cytokine (Mendes et al., 2019). A study on RL95-2 endometrial cells proved that the bioactive fraction of *Phaleria macrocarpa* is antiangiogenic and pro-apoptotic (Maharani et al., 2021). Meanwhile, more research is needed to study *Phaleria macrocarpa*, primarily related to the role of flavonoid extract on IL-17A levels compared to dienogest, so this study aims to compare the effect of administration of flavonoids crown of gods and dienogest on IL-17A levels in endometriosis model mice.

MATERIALS AND METHODS

Research Design

The research design used by the author in this study is true experimental with the post-test-only control group design approach. The samples used in this study were female mice aged 6-8 weeks. This study has passed an ethical review from Health Research Ethics Commission Brawijaya University No 67/EC/KEPK/03/2023.

Animal

The study used flavonoid extracts as ready-made extract preparations derived from the Mahkota Dewa fruit, taken from the whole flesh of the *Phaleria macrocarpa* Boerl fruit obtained from Langa City, Aceh Province, Indonesia. This study used healthy female mice (*Mus musculus*), aged 12 weeks old, weight 20-30 grams. A total of 35 mice were divided into seven groups, including healthy mice ($n = 5$), endometriosis mice ($n = 5$), endometriosis mice received *Phaleria macrocarpa* at a dose of 3.75mg/day ($n = 5$), endometriosis mice received *Phaleria macrocarpa* at a dose of 7.5 mg/day ($n = 5$), endometriosis mice received *Phaleria macrocarpa* at a dose of 11.25 mg/day ($n = 5$), mice model of endometriosis received *Phaleria macrocarpa* at a dose of 15 mg/day ($n = 5$) and ice model of endometriosis received dienogest at dose 0,0052 mg/day. Flavonoids of *Phaleria macrocarpa* fruit extract were administered orally for 14 days. Dienogest administration is given within 15 days with a dose of 0.0052 mg/day for experimental animals. The doses are given based on previous studies. Dienogest is given orally through a sonde or pipette.

Endometriosis Model

Mice models of endometriosis were according to a previous study (Sutrisno et al., 2017). This study used experimental animals as samples, namely endometriosis mice. Mice were given endometrial tissue from patients with adenomyosis; then, the tissue was injected intraperitoneally into each mouse. After the mice were injected with cyclosporine A, ethinyl estradiol, and endometrial tissue on the 14th day, they were expected to become mice models of endometriosis. As proof, on day 15, an immunohistochemical examination was performed to assess whether ER- α and ER- β were expressed in endometriosis lesions in the peritoneal tissue of mice.

Preparation Of Ekstrak

The study used flavonoid extracts as ready-made extract preparations derived from the Mahkota Dewa fruit, taken from the whole flesh of the *Phaleria macrocarpa* Boerl fruit obtained from Langa City, Aceh Province, Indonesia. Two thousand five hundred grams of *Phaleria macrocarpa* flour soaked in 30 L of 96% ethanol, then stirred (± 30 minutes) until well mixed. The mixture was then allowed to stand for five nights until settled. Next, filtering with the Buncher funnel gets the filtrate.

Preparation Of Flavonoid

The separation of flavonoid compounds to obtain n-hexane and n-butanol partitions. The ethanol extract was dissolved in n-hexane (1L). After obtaining a precipitate, the n-hexane solution is removed, and the ethanol precipitate is evaporated at a temperature of 45 C. Separation is continued with n-butanol. The ethanol solution was mixed with n-butanol (centrifuged with tool name biobased at 3000 RPM for 10 min). The supernatant is then taken and evaporated at 60° C to obtain a concentrated flavonoid solution.

Analysis of IL-17A

Peritoneal fluid isolation was performed according to the procedure in a previous study (Sutrisno et al., 2017). Before biomarker analysis, peritoneal fluid samples were stored at -80°C. Measurement of IL-17A levels using the Enzyme-linked immunosorbent assay (ELISA) method. IL-17A (Cat.No.KTE7012) with content components, namely 48 well strip microplate, antibody, sample diluent, assay buffer, streptavidin HRP, HRP substrate, stop solution, wash buffer, plate cover.

Statistic analysis

Data were presented as the mean \pm SD, and the ANOVA test and Post Hoc analyzed differences between treatment groups. The analysis was performed with the SPSS 26.0 statistical package for Windows programs. The probability values ($p <$

0.05) expressed significantly different. Furthermore, a post hoc test determines which group has the most significant difference.

RESULTS AND DISCUSSION

Based on the results of the immunohistochemical examination showed robust expression of REa and REb in the peritoneal tissue.

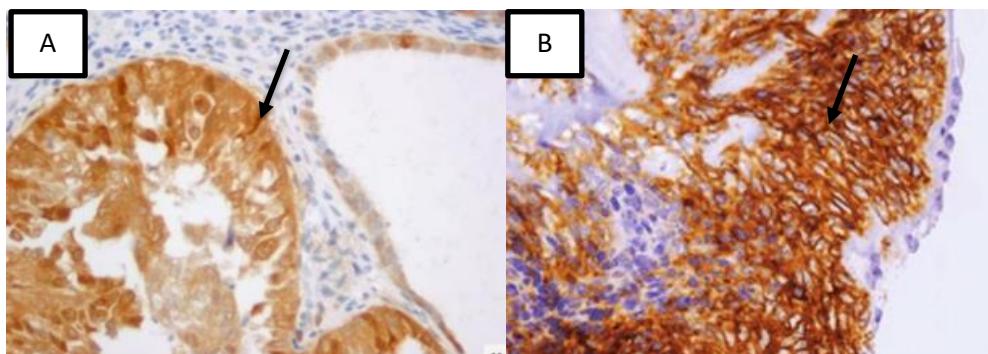


Figure 1. Expression of ER- α and ER- β in Endometriosis Mice Model. A= Strong expression of ER- α in the cytoplasm of stromal cells of endometriotic lesions of the peritoneal tissue is indicated by the presence of a chromogen brown color. B= Strong ER- β expression in the cytoplasm of stromal cells of endometriotic lesions of the peritoneal tissue is indicated by the presence of a chromogen brown color.

After proving that the mice had endometriosis, the mice in the treatment group (P1, P2, P3, P4) were given a dose of flavonoids of mahkota dewa fruit extract at a dose of 3.75 mg/mouse/day, 7.5 mg/mouse/day, 11.25 mg/mouse/day, P5 was given dienogest 0.0052 mg/mice/day. Based on research using One Way Anova, a p-value of 0.000 is obtained, which is smaller than $\alpha = 0.05$ ($p < 0.05$). It can be concluded that there is a significant effect of giving the crown of gods on IL-17A levels; In other words, the administration of Mahkota Dewa fruit extract was shown to reduce IL-17A levels in the endometriosis mice model.

Table 1. Effects of *Phaleria macrocarpa* and Dienogest on Levels IL-17A

Observational Groups	Mean\pmSD (ng/mL)	p-value
Healthy mice	24,34 \pm 14,05 ^a	
Endometriosis mice	245,33 \pm 15,1 ^d	
Endometriosis mice + <i>Phaleria macrocarpa</i> 3,75 mg dose	41,69 \pm 7,25 ^a	0,000
Endometriosis mice + <i>Phaleria macrocarpa</i> 7,5 mg dose	94,55 \pm 22,53 ^c	
Endometriosis mice + <i>Phaleria macrocarpa</i> 11,25 mg dose	88,04 \pm 31,79 ^{b,c}	
Endometriosis mice + <i>Phaleria macrocarpa</i> 15 mg dose	68,26 \pm 16,30 ^c	
Endometriosis mice + dienogest 0,0052 mg dose	106,31 \pm 18,62 ^b	

Description: Significance ($p < 0.05$) with One Way ANOVA test and post hoc test. On the average $IL-17A \pm SD$ if it contains different letters it means there is a

significant difference and if it contains the same letters it means there is no significant difference.

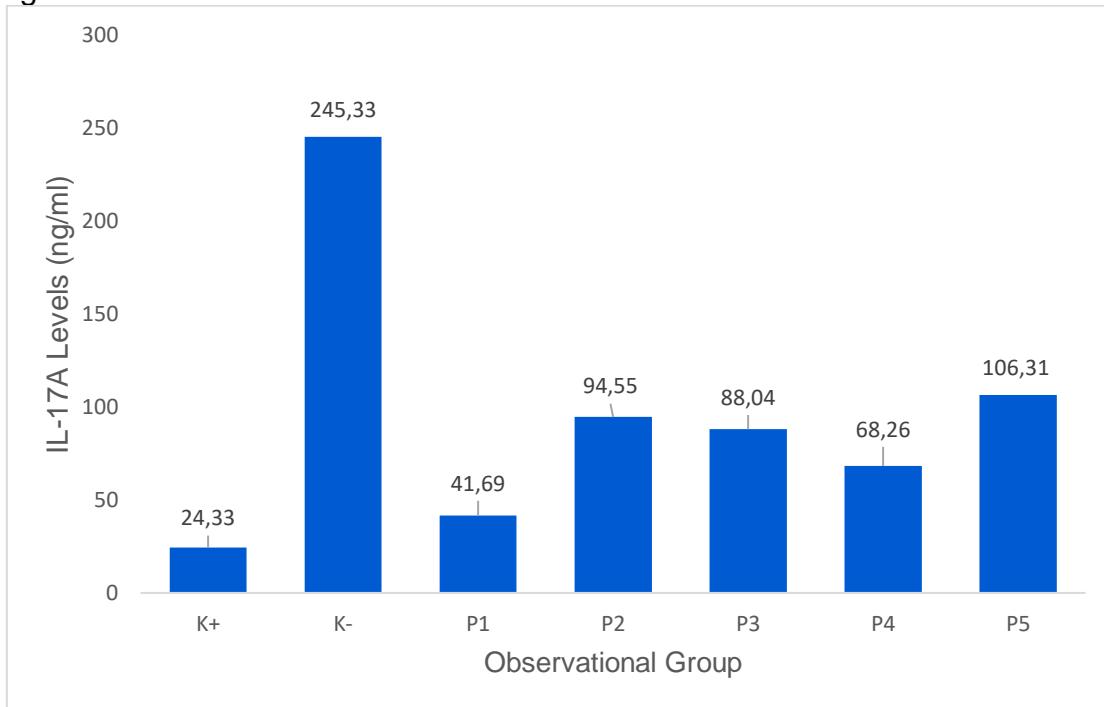


Figure 2. Groups with Different Letters on Histogram Bars are Statistically IL-17A Levels. K-: negative control group (healthy mice); K+: positive control group (endometriosis mice without treatment); P1: first treatment (endometriosis mice received *Phaleria macrocarpa* with a dose of 3,75 mg/day); P2: second treatment (endometriosis mice received *Phaleria macrocarpa* with a dose of 7,5 mg/day); P3: third treatment (endometriosis mice received *Phaleria macrocarpa* with a dose of 11,25 mg/day); P4: fourth treatment (endometriosis mice received *Phaleria macrocarpa* with a dose of 15 mg/day); P5: fifth treatment (endometriosis mice received dienogest with dose of 0,0052 mg/day).

Based on Figure 2 shows that there was a significant decrease in IL-17A levels in the negative control group compared to the positive control. The mean results showed that the positive controls had levels of 245,33 + 15.1, higher than the negative group, 24.34 + 14.05. Based on Table 1 and Figure 2, the results of giving a dose of flavonoids of mahkota Dewa fruit extract significantly reduced IL-17A levels in endometriosis mice. Flavonoids of *Phaleria macrocarpa* extract at a dose of 3.75 mg/mice/day reduced IL-17A levels most effectively compared to other doses. When compared to endometriosis mice that were given Dienogest 0.0052 mg/mice/day, endometriosis mice that were given the flavonoid of *Phaleria macrocarpa* fruit extract dose of 3.75 mg/mice/day had the lowest levels of IL-17A and significantly different.

Based on the results of statistical analysis using One-Way ANOVA (Table 1), it was found that there was a significant difference in the treatment of flavonoids from the mahkota dewa fruit extract and dienogest ($p<0.05$) towards decreasing IL-17A levels in endometriosis model mice.

The flavonoids of Mahkota dewa extract have higher results in reducing IL-17A levels than dienogest. Flavonoids are anti-inflammatory for immune cell modulation, which can inhibit cell activation, maturation, signaling transduction, and secretory processes, causing a decrease in the release of proinflammatory cytokines (Maleki et al., 2019). Flavonoids weaken proinflammatory cytokines (Mendes et al., 2019).

Flavonoids can interfere with the function of cells that mediate the inflammatory response, thereby interfering with the inflammatory process; flavonoids act as downregulation of myeloid cells (primarily macrophages), meaning that they decrease the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, proinflammatory cytokines (Ribeiro et al., 2015). In addition, it was stated that when the proinflammatory pathway is activated, cytokine-producing cells, including macrophages, will remove foreign bodies. However, if the body fails to remove these foreign bodies in the initial phase, inflammation will increase, mediated by increased production of cytokines and chemokines (Al-Khayri et al., 2022). The overexpression of proinflammatory cytokines as well as the TNF-stimulated gene-6 factor (TSG-6), in the endometrium of patients with endometriosis, was confirmed in a small cohort study conducted by (Matteo et al., 2017). It seems that cell communication between ectopic endometrial cells and healthy cells stimulates the alterations in cytokines expression and may be responsible for the cytokine profile in the peritoneal cavity of patients with endometriosis (Yoshino et al., 2019). Flavonoids, in their activity in the inflammatory response, act as suppressors of inflammatory mediators such as reactive oxygen species (ROS) and nitric oxide (NO); regulate the activity of inflammatory enzymes, such as cyclooxygenases (COXs) and nitric oxide synthase (iNOS); suppress production and expression of cytokines and modulation of transcription factors, such as nuclear factor κ-activated B-cell light chain enhancer (NF-κB) and activated protein-1 (AP-1) (Leyva-López et al., 2016).

Dienogest is a fourth-generation selective progestin that combines the pharmacological properties of a 19-nortestosterone and a progesterone derivative, providing a marked local effect on endometriotic lesions with little androgenic, estrogenic, glucocorticoid, or mineralocorticoid activity and minimal impact on metabolic parameters. (Andres et al., 2015). Dienogest influences the inflammatory response in endometrial tissue. These studies identified modulation of prostaglandin (PG) production and metabolism (PGE₂, PGE₂ synthase, cyclooxygenase-2, and microsomal PGE synthase-1), proinflammatory cytokines such as IL-17A, and production of chemokines interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α, monocyte chemoattractant protein-1 and stromal cell-derived factor, growth factor biosynthesis (vascular endothelial growth factor and nerve growth factor) and signaling kinases, which are responsible for controlling inflammation (Bedaiwy et al., 2017).

The results of this study found a significant difference should be determined by statistically the test shown in Table 1 obtained the results in levels between the negative control group 24.34 ± 14.05 and the positive control group 245.33 ± 15.10 . The results also found a significant difference in IL-17A levels between the positive control group 245.33 ± 15.10 and the treatment group with the provision of flavonoid extract of the crown of the god 3.75 mg. Further testing of the post hoc test found that treatment group 1 had a significant difference from the positive control group. Based on the results, it was found that a dose of 3.75 mg was the smallest dose that was most optimal for endometriosis therapy. This is also supported by the study by (Calabrese et al., 2010) on the concept of hormesis; stress agents, including drugs, toxins, and natural substances, when given in low doses, can cause a positive response in terms of adaptation or protection from stressors, while at higher concentrations, cause toxic effects.

Phaleria macrocarpa is a medicinal plant widely used in traditional Indonesian medicine to treat several diseases (Alara et al., 2016). This plant contains tannins, terpenoids, alkaloids, and flavonoids (Altaf et al., 2013). Flavonoids are secondary

metabolites found in plants, contribute to plant growth and development, and have prominent applications in food and medicine (Liu *et al.*, 2021).

Another study said that progesterone receptor-mediated inflammatory response inhibition in PR-expressing epithelial cells. It also shows that Dienogest inhibits the inflammatory response in stromal cells (Grandi *et al.*, 2016). His research stated that endometriosis patients who received dienogest experienced a significant increase compared to those who did not receive treatment (Hayashi *et al.*, 2012). This study did not conduct a toxicity test on the dose of Mahkota Dewa fruit extract before the study, so the maximum toxic dose on endothelial cells is unknown. Further research can be used as an alternative therapy for patients with endometriosis that is safe, inexpensive, and has minimal side effects. It is hoped that future researchers will not only look at IL-17A levels but also assess IL-17A expression so that they can find out more clearly the decrease in IL-17A cytokines in peritoneal fluid.

CONCLUSION

Flavonoid fruit extract *Phaleria macrocarpa* can reduce IL-17A levels in endometriosis mice model. This study shows that the flavonoid extract of Mahkota dewa can be used as a potential anti-inflammatory therapy by restoring the anti-inflammatory balance (IL-17A).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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