



Original Research

Effect of *Phaleria macrocarpa* Extract on NF-KB, MMP-2, and MMP-9 Expression in Endometriosis Mice Model

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KEYWORD

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ABSTRACT

Introduction: Endometriosis is the most common disease that affects the reproductive health and function of women of reproductive age. Treatment for endometriosis includes surgery and medication. *Phaleria macrocarpa* is a plant native to Indonesia that contains bioactive fractions known to have antiproliferative and apoptotic effects. Therefore, this study aimed to evaluate the effect of *Phaleria macrocarpa* extract on matrix metalloproteinases (MMP-2 and MMP-9) and NF-KB expression in the endometriosis mice model (*Mus musculus*).

Methods: This study used a Randomized Post-Test Only with Control Group Design. Involves six groups, namely a negative control group (healthy mice without flavonoids from *Phaleria macrocarpa* fruit extract), a positive control group (an endometriosis model without being given flavonoids from *Phaleria macrocarpa* fruit extract), and a treatment group, namely a group given four different doses of flavonoids from *Phaleria macrocarpa* fruit extract: 3.75 milligrams per day, 7.5 milligrams per day, 11.25 milligrams per day and 15 milligrams per day. expressions of NF-KB, MMP-2, and MMP-9 were seen using Immunohistochemistry staining and observed under a microscope with 40x magnification. The data collection used an immunoratio technique with ImageJ analysis software. Furthermore, data analysis using the one-way ANOVA method.

Results: In this study, the treatment group with four different doses of *Phaleria macrocarpa* extract could reduce the expression of MMP-2, MMP-9, and NF-KB. Groups with higher doses of *Phaleria macrocarpa* extract showed lessen of MMP-2, MMP-9, and NF-KB expression.

Conclusion: In the endometriosis mice model, *Phaleria macrocarpa* fruit can reduce NF-KB, MMP-2, and MMP-9 expression.

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INTRODUCTION

Endometriosis is the most common disease that affects the reproductive health and function of women of reproductive age. The prevalence of endometriosis reaches 6-10% in childbearing-age women. Approximately 50-60% of these women experience pelvic pain and infertility problems. There are few ectopic changes in the endometrium in women with endometriosis compared to average women. The prevalence of endometriosis in women of reproductive

age ranges from 3-10% and contributed to 9-50% in infertile women. In the group undergoing operative evaluation procedures for dysmenorrhea, the prevalence of endometriosis reaches 60%. Half of the patients with endometriosis show clinical symptoms of pelvic pain and infertility, reducing women's quality of life. If not getting the proper treatment, the impact and progression of endometriosis will continue throughout a woman's life [1–4]. The development of endometrial lesions involves immunosuppressive factors, abnormal communication between the peritoneum and the ectopic

endometrium, and activation of exosomes [5]. There is a change in the phenotype of peritoneal mesothelial cells in the form of ectopic endometrial cells to attach and attack targets resulting in damaged peritoneal signaling pathways [6]. Peritoneal mesothelial cells will release pro-inflammatory cytokines and growth factors to stimulate angiogenesis and endometriosis cell proliferation [7].

Treatment for endometriosis includes surgery and medication. This therapy has many disadvantages, including expensive, only pain relief, and treatment is carried out continuously because endometriosis can recur. It is known that endometriosis has a strong expression of estrogen receptor (ER)- α , ER- β , and progesterone receptor (PR) [8]. Herbal therapy is still being developed today, one of which is derived from *Phaleria macrocarpa* [9,10]. *Phaleria macrocarpa* is a native Indonesian valuable plant as food and medicine. This plant contains tannins, terpenoids, alkaloids, and flavonoids [11].

A study on endometrial cells proved that the bioactive fraction of *Phaleria macrocarpa* is antiangiogenic, antioxidant, anti-inflammatory, anti-cancer, and pro-apoptotic [12,13]. Another study showed that *daidzein-rich isoflavone aglycone* (DRIA) in *Phaleria macrocarpa* inhibits cell proliferation in endometriosis, making it a potential therapeutic option for the management of endometriosis, in addition, a study mentioning the effect of NF-KB inhibitors in ECs and promoting endometriotic cells (ECCS) has shown that inhibition of NF-KB can reduce the development and maintenance of endometriosis [14,15].

In this case, the role of bioactive substances, namely flavonoids from *Phaleria macrocarpa*, against endometriosis is still being developed, with published research being significant to reveal its pathophysiology. Therefore, this study aimed to evaluate the effect of extracts of the fruit of the crown god on matrix metalloproteinase and NF-KB in endometriosis model mice.

MATERIAL AND METHODS

Study Design

This study uses an actual experimental design carried out in the laboratory in vivo on female mice (*Mus musculus*) with a Randomized Post-Test Only with Control Group Design research design; each animal has the same probability of receiving treatment. The research was carried out at the Embryology Laboratory of the Faculty of Veterinary Medicine, Airlangga University, Surabaya, to maintain mice and terminate mice. Furthermore, the examination of the expression of MMP-2, MMP-9, and NF-KB in the Laboratory of Physiology, Faculty of Medicine, Universitas Brawijaya Malang. The study was carried out for approximately

seven weeks with a period of adaptation to acclimatization for one week, injection of Methyl Prednisolone 0.5 milligrams per kilogram of body weight for one week, then implantation of adenomyosis tissue in the peritoneal mice. The process continued by administration of Ethynil Estradiol injection to the mice model, endometriosis mice model formed then given flavonoids extract of the God's crown. Various doses for 14 days, followed by termination of experimental animals. Furthermore, samples were made with immunohistochemical staining.

Sample

This study used a sample of 36 healthy adult female *Mus musculus* mice, aged 18-20 days, weighing 20-30 grams, active and anatomically healthy from the Embryology Laboratory of the Faculty of Veterinary Medicine Airlangga University, Surabaya. These mice were chosen as research samples because they are easy to maintain and are relatively healthy animals. They are suitable for use in various types of experimental research, and their immunological response can be observed. Therefore, the inclusion criteria of the research subjects were mice (*Mus musculus*) of the female sex, aged 60-90 days, with a body weight of 20-30 grams, and in a healthy condition characterized by active movements. Meanwhile, the exclusion criteria were if the mice looked sick (not moving) before treatment or died during the research process.

Randomized and Interventions

This study involved six groups, namely a negative control group (healthy mice without flavonoids from God's crown fruit extract), a positive control group (endometriosis mice without flavonoids from God's crown fruit extract), and a treatment group, namely a group given various doses of flavonoids from God's crown fruit extract. different: 3.75 milligrams per day, 7.5 milligrams per day, 11.25 milligrams per day, and 15 milligrams per day. The dose is based on a previous study by Maharani *et al* (2021) on the phytochemical characteristics of the *Phaleria macrocarpa* and its activity in inhibiting the occurrence of peritoneal endometriosis.

Ethics

All techniques in this study were carried out in compliance with the appropriate manuals and regulations and were approved by the Health Research Ethics Committee, Faculty of Medicine, Brawijaya University, Malang, Indonesia with ethic code number: 106/EC/KEPK/04/2021.

Statistical Analysis

Statistical analysis was analyzed using IBM SPSS Version 26.0 for Windows. Furthermore, data analysis was carried out on the effect of the dose of *Phaleria*

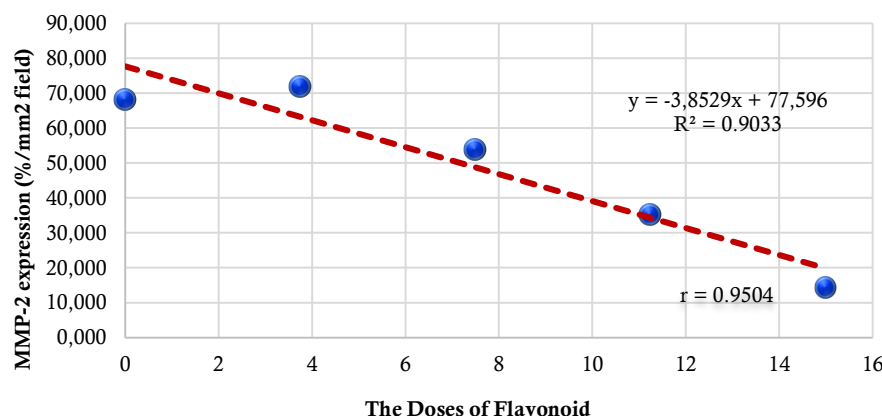


Fig. 1. The Effect of the *Phaleria macrocarpa* Extract on MMP-2 Expression in Endometriosis Mice Model

macrocarpa fruit extract on the expression of NF-KB, MMP-2, and MMP-9, starting with testing the normality of the residual data on the effect of the dose of *Phaleria macrocarpa* extract with Shapiro Wilk, testing the homogeneity of the data with the Levene test and testing the difference in the effect of the dose of the crown extract. One Way ANOVA (F test) and linear regression test that been used between the dose of God's crown fruit extract and the research parameters (NF-KB, MMP-2, and MMP-9).

Fig. 3 shows a correlation between the dose of *Phaleria macrocarpa* fruit extract and NF-KB expression can be represented through a regression line to determine the effect of the dose of *Phaleria macrocarpa* fruit extract with NF-KB expression with the equation $y = -5.193 X + 82.096$ and the correlation coefficient ($r = 0.9751$ (r table = 0.811) $< r$ the table so that there is a non-significant correlation between the dose of *Phaleria macrocarpa* fruit extract and the expression of NF-KB. The correlation coefficient of 0.9751 is 0.80 – 0.99, indicating a solid correlation between the dose of extracts of *Phaleria macrocarpa* fruit extract and the expression of NF-KB. On the other hand, the equation

shows that the magnitude of the expression of NF-KB in the absence of a dose of an extract of *Phaleria macrocarpa* fruit is 82.096%/mm² field of view. Meanwhile, each additional dose of *Phaleria macrocarpa* fruit extract, as much as 1 mg, will reduce the expression of NF-KB by 5.193%/mm² field of view. This means that the linear correlation is very representative of the effect of the dose of *Phaleria macrocarpa* fruit extract with the expression of NF-KB with a solid level of correlation.

RESULTS

This study uses an experimental design (true experimental) carried out in the laboratory in vivo on female mice (*Mus musculus*) with a Randomized Post-Test Only with Control Group Design. Each animal has the same probability of receiving treatment. Involves six groups, a negative control group (healthy mice without flavonoids from *Phaleria macrocarpa* fruit extract), a positive control group (endometriosis mice without flavonoids from *Phaleria macrocarpa* fruit extract), and a treatment group (The treatment groups were given

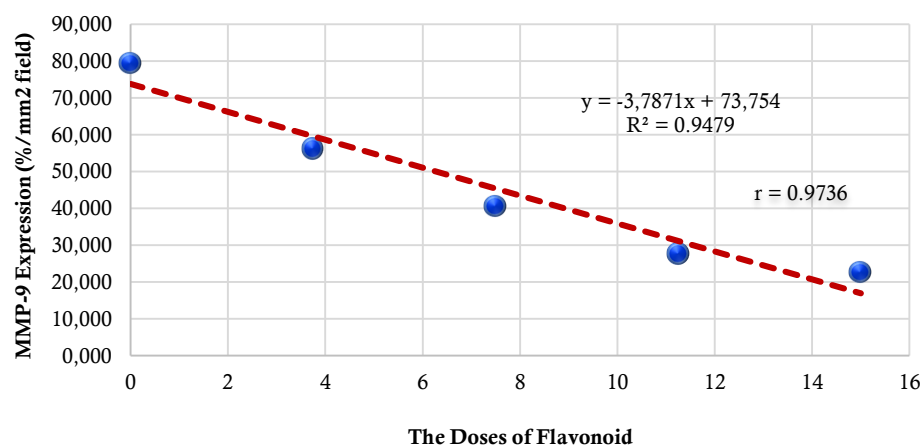


Fig. 2. The Effect of the *Phaleria macrocarpa* Extract on MMP-9 Expression in Endometriosis Mice Model

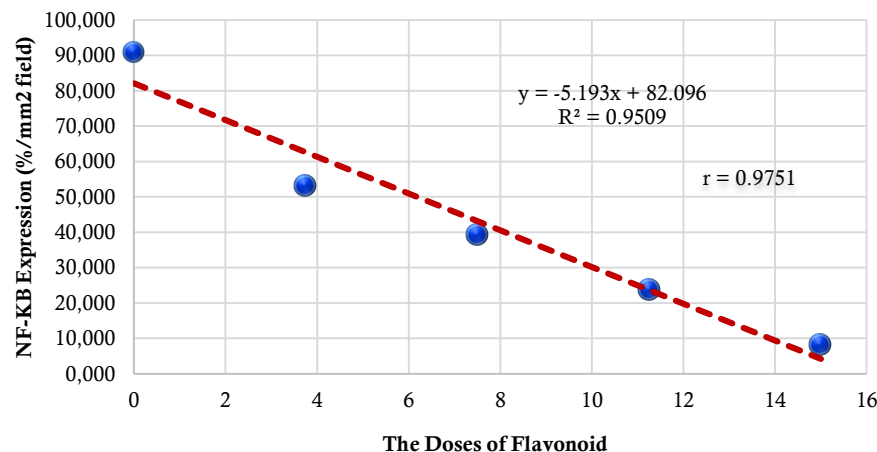


Fig. 3. The Effect of the *Phaleria macrocarpa* Extract on NF-KB Expression in Endometriosis Mice Model

various extracts of *Phaleria macrocarpa* fruit extraction on doses of 3.75 milligrams per day, 7.5 milligrams per day, 11.25 milligrams per day, and 15 milligrams per day).

Fig. 1 shows the correlation between the dose of the *Phaleria macrocarpa* fruit extract and MMP-2 expression can be represented through a regression line to determine the effect of the dose of the *Phaleria macrocarpa* fruit extract with MMP-2 expression with the equation $y = -3.8529 X + 77.596$ and the correlation coefficient $(r) = 0.9033$ ($r \text{ table} = 0.811$) $< r \text{ table}$ so that there is a non-significant correlation between the dose of the *Phaleria macrocarpa* fruit extract and MMP-2 expression. The correlation coefficient of 0.9033 is 0.80 – 0.99, indicating a solid correlation between the dose of extracts of the *Phaleria macrocarpa* fruit extract and the expression of MMP-2. On the other hand, the equation shows that the magnitude of MMP-2 expression without a dose of an extract of the *Phaleria macrocarpa* fruit is 77.596 %/mm2 field of view. Meanwhile, each additional dose of an extract of the *Phaleria macrocarpa* fruit of 1 milligram will reduce MMP-2 expression by 3.853%/mm2 field of view. This means that the linear correlation strongly represents the effect of the dose of *Phaleria macrocarpa* fruit extract with MMP-2 expression with a solid level of correlation.

Fig. 2 shows a correlation between the dose of *Phaleria macrocarpa* fruit extract and MMP-9 expression can be represented through a regression line to determine the effect of the dose of *Phaleria macrocarpa* fruit extract with MMP-9 expression with the equation $y = -3.7871 X + 73.754$ and the correlation coefficient $(r) = 0.9736$ ($r \text{ table} = 0.811$) $< r \text{ table}$ so that there is an insignificant correlation between the dose of extracts of the crown of the gods with the expression of MMP-9. The correlation coefficient of 0.6864 is 0.80 – 0.99, indicating a solid correlation between the dose of extracts of the *Phaleria macrocarpa* fruit extract and the expression of MMP-9. On the other hand, the equation

shows that the magnitude of the expression of MMP-9 in the absence of a dose of an extract of *Phaleria macrocarpa* fruit is 73.754%/mm2 field of view. Meanwhile, each 1 mg additional dose of *Phaleria macrocarpa* fruit extract will reduce the expression of MMP-9 by 3.787%/mm2 field of view. This means that the linear correlation is very representative of the effect of the dose of *Phaleria macrocarpa* fruit extract with MMP-9 expression with a solid level of correlation.

Fig. 3 shows a correlation between the dose of *Phaleria macrocarpa* fruit extract and NF-KB expression can be represented through a regression line to determine the effect of the dose of *Phaleria macrocarpa* fruit extract with NF-KB expression with the equation $y = -5.193 X + 82.096$ and the correlation coefficient $(r) = 0.9751$ ($r \text{ table} = 0.811$) $< r \text{ table}$, thus there is the non-significant correlation between the dose of *Phaleria macrocarpa* extract and the expression of NF-KB. The correlation coefficient of 0.9751 is 0.80 – 0.99, indicating a solid correlation between the dose of extracts of *Phaleria macrocarpa* extract and the expression of NF-KB. On the other hand, the equation shows that the magnitude of the expression of NF-KB in the absence of a dose of an extract of *Phaleria macrocarpa* fruit is 82.096%/mm2 field of view. Meanwhile, each 1 mg additional dose of *Phaleria macrocarpa* extract will reduce the expression of NF-KB by 5.193%/mm2 field of view. This means that the linear correlation is very representative of the effect of the dose of *Phaleria macrocarpa* extract on the expression of NF-KB with a solid level of correlation.

DISCUSSION

Several previous studies have stated that endometriosis is a collection of multi-factors including immune, genetic and hormonal environments characterized by abnormal expression of inflammatory

factors. An essential step in developing endometriosis is the association between inflammation and activation of the aromatase gene in the endometrium, followed by local production of estrogen in the endometrium. In addition, an estrogenic microenvironment has been reported to activate macrophages in the peritoneum with a consequence in the secretion of pro-inflammatory cytokines such as tumor necrosis factor- (TNF- α) and interleukin-1 β (IL-1 β), which will activate NF-KB [17].

Nuclear Factor-kappa β (NF-KB) is a transcription factor that stimulates the process of cell survival, proliferation, and differentiation and is a factor that supports aromatase expression and inflammation in endometriosis. The first step in the inflammatory process is activating and translocating NF-KB from the cytoplasm to the cell nucleus. NF-KB can activate several pro-inflammatory cytokines such as TNF-, IL-1 β , IL-8, and other inflammatory mediators. In addition, NF-KB also triggers the activation of Angiotensin II receptor type 1 (AGTR1), stimulating cell migration and proliferation and inhibiting endometrial stromal cell apoptosis [17,19].

Meanwhile, Endometrium in endometriosis has increased the expression of specific groups of proteolytic enzymes, namely Matrix Metalloproteinase (MMP) and Tissue Inhibitor Matrix Metalloproteinase (TIMP), resulting in the implantation of ectopic endometrial cells. A misregulated state can contribute to the formation of ectopic endometriotic lesions in the peritoneal cavity. An increase in MMP-2 and MMP-9 is associated with an increase in the extent of endometrial lesions, especially in the peritoneal cavity. The hormone progesterone can inhibit the secretion of MMP. Therefore, in tissues that are less sensitive to the hormone progesterone, increased cytokine activity will stimulate increased MMP activity in endometriosis and facilitate invasion, resulting in the formation of endometrial lesions. MMP-9 secretion is caused by the activation of the release of Reactive Oxygen Species (ROS) in the peritoneal cavity and causes oxidative stress to increase [7].

Research by Wiweko et al (2015) showed that the bioactive substances contained in *Phaleria macrocarpa* significantly reduce pain in patients with endometriosis and/or adenomyosis. This is related to the activity of these substances as an anti-inflammatory to reduce pain without being accompanied by a hypo-estrogenic state. The anti-inflammatory activity of *Phaleria macrocarpa* was found to have strong anti-inflammatory activity due to its content, including terpenoids, saponins, tannins, and flavonoids. This activation leads to changes involving gene transcription factors including nuclear factor kappa- β (NF-KB). This is explained in the research of Takaoka et al. (2018) and research by Wei and Shao (2018), namely flavonoid preparations can inhibit the activation of NF-KB by inhibiting the activity

of aromatase inhibitors associated with the development of endometrial lesions so that the presence of NF-KB in the endometrium supports its role in the physiology and pathophysiology of endometrial cells. When NF-KB decreases, the development of endometriosis will decrease. In endometriosis, there is also an increase in the expression of a specific group of proteolytic enzymes, namely Matrix Metalloproteinase (MMP) in addition to Tissue Inhibitor Matrix Metalloproteinase (TIMP), resulting in the implantation of ectopic endometrial cells. Misregulation of MMP synthesis and secretion from endometrial lesions combines with TIMP-1 amounts in the peritoneal fluid, thereby altering the functional matrix components surrounding the peritoneal fluid, inducing aggressive behavior and facilitating ectopic cell invasion. In 2015 research by Wiweko et al. revealed that giving a regimen of *Phaleria macrocarpa* with the trademark Dismeno, a 100 mg preparation in patients with endometriosis or accompanied by adenomyosis for eight days can reduce pain levels according to the visual analog scale (VAS) [16]. In 2017, the consensus on the management of pain in endometriosis, the first revision by the Indonesian Association of Reproductive Endocrinology and Fertility, stated that giving *Phaleria macrocarpa* bioactive extract three times a day at a dose of 100 mg was effective for reducing pain in dysmenorrhea, pain before menstruation, dyschezia during menstruation and dysuria. This study found a decrease in one of the agent parameters and inflammatory factors (NF-KB, MMP 2, and MMP 9) that affect the formation of endometriosis cells and their development. So that the treatment with the crown of the gods is also expected to inhibit the formation of endometriosis lesions and reduce pain symptoms caused by endometriosis [20].

The study used endometriosis model mice from previous research, endometriosis model mice were evaluated visually by looking at endometriosis lesions and the expression of estrogen receptors (ER) in peritoneal tissue. According to previous studies, endometriosis model mice were found to have a predominance of ER and peritoneal tissue [8].

In this study, the effect between the dose of *Phaleria macrocarpa* extract and NF-KB expression was found that there was a solid correlation with each addition of 1 mg of *Phaleria macrocarpa* extract dose would decrease NF-KB expression. Research, stated that the extract of the *Phaleria macrocarpa* contains some phytochemical compounds in some parts that play a role as antioxidant, anti-inflammatory, and anti-cancer properties So that the phytochemical cause of the expression of inflammatory parameters decreased in the administration of *Phaleria macrocarpa* extract in this study, although the pathogenesis and pathophysiology of endometriosis are not fully understood. In one study, it was stated that the overexpression of the estrogen

receptor (ER- β) in endometrial tissue has a role in triggering the occurrence and development of endometriosis and endometriosis pain. Increased expression of estrogen receptors (ER- β) will suppress the expression of estrogen receptors- α (ER- α), increasing the ratio of ER- β /ER- α . Increased ER- β can also trigger suppression of the progesterone receptor and will trigger cyclooxygenase-2 (COX-2) activity, which will cause progesterone resistance and an inflammatory reaction. *Phaleria macrocarpa* is known to suppress the overexpression of the genes ER- β , COX-2, and phospholipase A2 (cPLA2). In addition, the use of *Phaleria macrocarpa* bioactive extract can also increase the regulation of PR gene expression that can lead to decreased expression of inflammatory parameters including NF-KB, MMP-2, and MMP-9 [15,21,22].

CONCLUSION

Phaleria macrocarpa can reduce the expression of NF-KB, MMP-2, and MMP-9 parameters in the endometriosis mice model. Therefore, macrocarpa can be used as an alternative to reduce inflammation in endometriosis.

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CONFLICT OF INTEREST

The authors declared that there was no conflict of interest regarding the publication of this article.

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