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The Effect of Flavonoid Extract from *Phaleria macrocarpa* to Proliferating Factors (MMP-1, MMP-3, MMP-7) in Endometriosis Mice Model

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ABSTRACT

Introduction: Endometriosis is an inflammatory disease influenced by estrogen characterized by ectopic growth of the endometrial stroma and glands. Matrix metalloproteinase (MMP) is a proteolytic enzyme that has an important role in the remodeling of the extracellular matrix in normal tissues but also contributes to pathologies such as tumor invasion. The therapy has many drawbacks, including being expensive and in need of herbal therapy as an alternative. *Phaleria macrocarpa* is a native plant of Indonesia that contains flavonoids. This study aimed to evaluate the effect of *Phaleria macrocarpa* flavonoid isolate on the development of apoptosis, proliferation, and angiogenesis in mice model endometriosis.

Material and Methods: This research is a true experimental study with a Randomized Post-Test Only with a Control Group in the laboratory. Samples are divided into six groups, a control group and an intervention group, administered with flavonoid extract from *Phaleria macrocarpa.* Data analysis was carried out by using the Independent T-Test with SPSS for Windows 19.0 software.

Results: Each control and intervention group consisted of 6 mice. The normality test for each variable shows p> 0.05. Administration of flavonoid in each group shows a significant decrease in MMP-1 (20.4 ± 7.74 vs 65.68 ± 10.97, p=0.000), MMP-3 (53.34 ± 9.66 vs67.47 ± 10.05, p=0.000), and MMP-7 expression (40.52 ± 5.43 vs 54.13 ± 4.08, p=0.000).

Conclusion: Flavonoids from the *Phaleria macrocarpa* fruit extract were able to reduce the expression of MMP-1, MMP-3, and MMP-7 in mice model endometriosis.

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INTRODUCTION

Endometriosis is classically defined as a chronic gynecological disease characterized by endometrial tissue appearing outside the uterus and thought to result from retrograde menstruation. However, this description is outdated and no longer reflects the true scope and manifestations of the disease. The clinical picture of endometriosis varies, namely the presence of heterogeneous pelvic lesions and manifestations of the disease outside the female reproductive tract. Endometriosis is now considered a systemic disease [1].

The incidence of endometriosis is still quite high, ranging from 6-10% in women of productive age from all racial and social groups [2]. Women with endometriosis lesions have a high risk of developing ovarian cancer and breast cancer, melanoma, asthma, rheumatoid arthritis, and cardiovascular disease [3].

The diagnosis of endometriosis may be very complex and sensitive to external factors, as it usually takes 7-10 years for a woman to be diagnosed with the condition. Endometriosis is also a heterogeneous condition, and more recent literature suggests that race/ethnicity can influence disease severity, i.e., Asian

women are more likely to be diagnosed with stage III/IV endometriosis compared to white women [4].

Endometriosis is an inflammatory disease influenced by estrogen characterized by ectopic growth of the endometrial stroma and glands accompanied by pelvic pain, and as much as 50% is detected in women undergoing infertility treatment [3]. The ectopic endometrium shows proliferation, implantation, and angiogenesis and may survive in different environments. Endometriosis is a condition in which there is endometrial tissue outside the uterus and is a benign gynecological condition. However, it can be aggressive. The pathology of endometriosis is complicated and of multi-factor origin. Until now, the pathophysiology and etiology of endometriosis have not been widely known, although several theories have been revealed [5, 6].

The development of endometriosis lesions involves immunosuppression factors, abnormal communication between the peritoneum and ectopic endometrium, and exosome activation [7]. There is a change in phenotype in peritoneal mesothelial cells in the form of ectopic endometrial cells to attach and attack the target, resulting in damage to the peritoneal pathway signal [8]. Peritoneal mesothelial cells will release proinflammatory cytokines and growth factors and become a stimulus for angiogenesis and endometriosis cell proliferation [6].

Matrix metalloproteinase (MMP) is a proteolytic enzyme that has an important role in the remodeling of the extracellular matrix in normal tissues but also contributes to pathologies such as tumor invasion. There are many MMP enzyme expressions, including MMP-1, MMP-3, and MMP-7, which are thought to play a role in the genetic expression of endometriosis and tumor metastasis [9–11]. The endometrium in endometriosis has increased expression of a specific group of proteolytic enzymes, namely Matrix Metalloproteinase (MMP) and Tissue Inhibitor Matrix Metalloproteinase (TIMP), resulting in implantation of ectopic endometrial cells. Misregulation of MMP synthesis and secretion from endometriosis lesions joining a certain amount of TIMP-1 in the peritoneal fluid then alters the components of the functional matrix around the peritoneal fluid, induces aggressive behavior, and facilitates ectopic cell invasion [10].

Endometriosis therapy includes surgery and medication. The therapy has many drawbacks, including being expensive, only relieving pain, and treatment is carried out continuously because endometriosis can recur [3]. Herbal therapy is needed from plants that are natural; the price is lower because it can be cultivated, is easy to obtain, and the expected side effects are minimal compared to synthetic anti-cancer drugs.

Phaleria macrocarpa is a native plant of Indonesia that is useful as food and medicine. This plant contains tannins, terpenoids, alkaloids, and flavonoids [12]. Previous studies have shown that standardized extracts are anti-proliferative and antiapoptotic Maharani et al., 2021 [13]. *Phaleria macrocarpa* ethanol extract triggers apoptosis of colon cancer cells [14]. A study on RL95-2 endometrial cells proved that the bioactive fraction of *Phaleria macrocarpa* is anti-angiogenic and pro-apoptotic [15]. Research by Takaoka et al. (2018) explained that flavonoid preparations can inhibit the activation of NF-KB and PGE2 by inhibiting aromatase inhibitor activity and expression of COX-2 to inhibit the formation of endometriosis lesions in vivo. The role of bioactive substances, in this case flavonoids from the *Phaleria macrocarpa*, against endometriosis is still small, so published research is very important to reveal its pathophysiology.

Therefore, this study aimed to evaluate the effect of *Phaleria macrocarpa* flavonoid isolate on the development of apoptosis, proliferation, and angiogenesis in mice model endometriosis. It is expected that giving the *Phaleria macrocarpa* to mice model endometriosis will affect cell proliferation and inflammatory reactions in endometriosis.

MATERIAL AND METHODS

This research is a true experimental study conducted in the laboratory in vivo on female mice (Mus musculus) with a research design of Randomized Post-Test Only with Control Group. The endometriosis mouse model was created using an acclimatization period of 1 week, injection of Methyl Prednisolone 0.5 mg/kg BW for one week, then implantation of adenomyosis tissue in the mice's peritoneum and administration of Ethynil Estradiol injection. Females were divided into six groups. Namely, the first group was a negative control group (healthy mice without the flavonoid extract), the second group was the positive control (endometriosis mice model with flavonoid), and the intervention group with four different dosages of flavonoid extract (3,75 mg/day, 7,5 mg/day, 11,25 mg/day dan 15 mg/day).

Mice were obtained from the Physiology Laboratory of the Faculty of Medicine, Universitas Brawijaya Malang, with the following conditions: female sex, age 18-20 days, weight 20-30 grams, healthy conditions characterized by active movements. Flavonoids were extracted from *Phaleria macrocarpa* with 96% ethanol, butanol, and centrifuge. The independent variable in this study is the different dosages of flavonoid extract given to the mice. The dependent variable in this study is the expression of MMP-1, MMP-3, and MMP-7. Data analysis with SPSS software for Windows 19.0.

Table 1. Comparison Expression of MMP-1, MMP-3, and MMP-7 Between Each Group

	MMP-1				MMP-3				MMP-7			
	mean	**±**	**sd. dev.**	*	**mean**	**±**	**sd. dev.**	*	**mean**	**±**	**sd. dev.**	*
K-	28.88	±	9.01	a	28.35	±	5.85	a	14.71	±	2.19	b
K+	65.68	±	10.97	c	67.47	±	10.05	c	54.13	±	4.08	d
P1	20.4	±	7.74	a	53.34	±	9.66	bc	40.52	±	5.43	c
P2	21.62	±	1.56	a	26.9	±	6.36	a	37.86	±	2.25	c
P3	39.96	±	11.7	ab	54.7	±	10.28	bc	13.05	±	2.47	b
P4	50.59	±	14.43	bc	49.89	±	6.72	b	6.21	±	2.66	a
p-value	0.000				0.000				0.000			

If mean ± sd contains different letters, it means there is a significant difference ($p < 0.05$), and if it contains the same letters, it means there is no significant difference ($p > 0.05$). * = Post-hoc Tukey test results

Table 2. Regression Analysis Results

Dependent Variable	Regression Equation	p-value	Correlation and p-value	R-Square
MMP-1	Y1 = 41.772 - 0.28321 X	0.715	r = -0.077 p = 0.715	0.59%
MMP-3	Y2 = 57.221 - 0.901 X	0.130	r = -0.311 p = 0.13	9.69%
MMP-7	Y3 = 55.02 - 3.289 X	0.000	r = -0.957 p = 0,000	91.67%

Y1: MMP-1 Expression, Y2: MMP-3 Expression, Y3 : MMP-7 Expression, Y3: Apoptosis Expression, X: Flavonoid Concentration Extract

RESULTS

This study used mouse models of endometriosis. Mice are left in a cage for one week to acclimatize to their environment. Next, mice are grouped into 6 groups, with each group consisting of 4 mice with two additional mice as a sample error. One group was the positive control group (K+), and 4 groups were the treatment group (P). The treatment group was given four doses: *Phaleria macrocarpa* extract 3.75 mg, 7.5 mg, 11.25 mg, and 15 mg for 14 days. Then, terminated mice and sampling of peritoneal fluid and peritoneal lesions were taken; after completion, the mice were buried (planted) in the ground to avoid environmental pollution. Each treatment was replicated five times. Naming and painting of immunohistochemical substances were observed, and the expression of MMP-1, MMP-3, and MMP-7 was observed with a microscope. Data collection techniques using immunoratio techniques with ImageJ analysis software. Table 1 reported the different expressions of MMP-1, MMP-3, and MMP-7 between each group.

Based on the results of the data normality test using the Levene test, the p-value of each group for each variable is greater than 0.05 ($p>0.05$), so it can be concluded that the data for all variables in each group (K-, K+, P1, P2, P3, P4) have an equal distribution. Normal, so that it can be continued with testing using the ANOVA test followed by the post-hoc Tukey test**.** Based on the results of analysis using ANOVA shown in Table 1, a p-value of 0.000 was obtained, smaller than $\alpha = 0.05$ ($p<0.05$). Thus, from this test, it can be concluded that there is a significant effect of giving flavonoid extracts from *Phaleria macrocarpa* on MMP1, MMP-3, and MMP-7 expression. In other words, there is a significant difference in MMP1, MMP-3, and MMP-7 expression due to the administration of flavonoid extracts from *Phaleria macrocarpa* with different doses.

Based on the results of the 5% Tukey test in Table 1 above, it was shown that the negative control group (K-) had an average MMP-1 expression of 28.88 ± 9.01. Statistically, it showed a significant difference with the positive control group (K+). This is shown from the average value of ± sd in groups K- and K+, which contain different letters. Administration of flavonoid extracts from *Phaleria macrocarpa* in groups P1, P2, and P3 resulted in lower MMP-1 expression K+. This proved that administration of flavonoid extracts in groups P1, P2, and P3 significantly decreased MMP-1 expression. However, administration of flavonoid extracts from the *Phaleria macrocarpa* fruit in the P4 group resulted in MMP-1 expression that was relatively the same as the K + group. This is shown from the average value of ± sd in group P4, which contains the same letter as group K+. It was also shown that the positive control group (K+) had the highest average MMP-3 expression of 67.47 ± 10.05 and was statistically significantly different from the negative control group (K-). This is shown from the average value of ± elementary school in groups K- and K+, which contain different letters. Administration of flavonoid extracts from *Phaleria macrocarpa* fruit at all dose levels resulted in higher MMP-3 expression than in the K+ group. A significant decrease was obtained in the P2 and P4

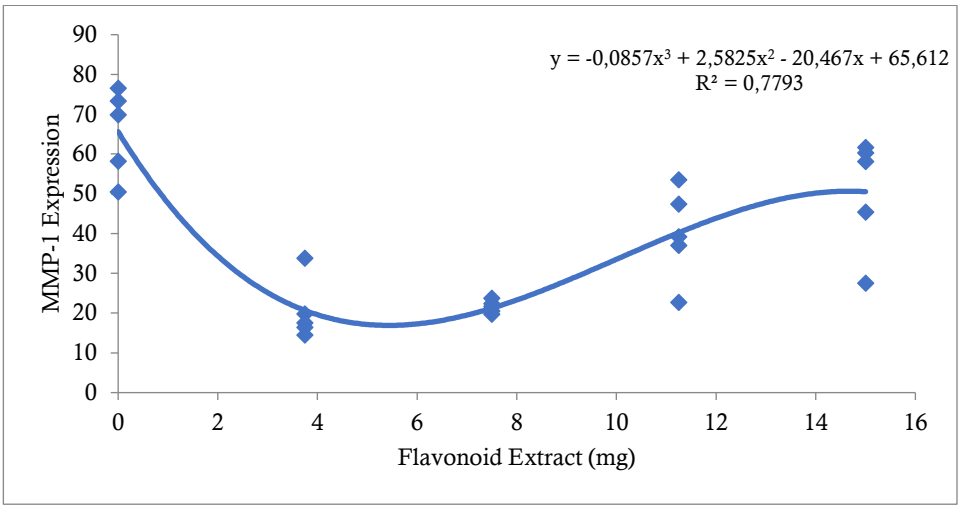

Fig. 1. Scatter Plot: Effect of Flavonoid Extract on MMP-1 Expression by Polynomial Regression

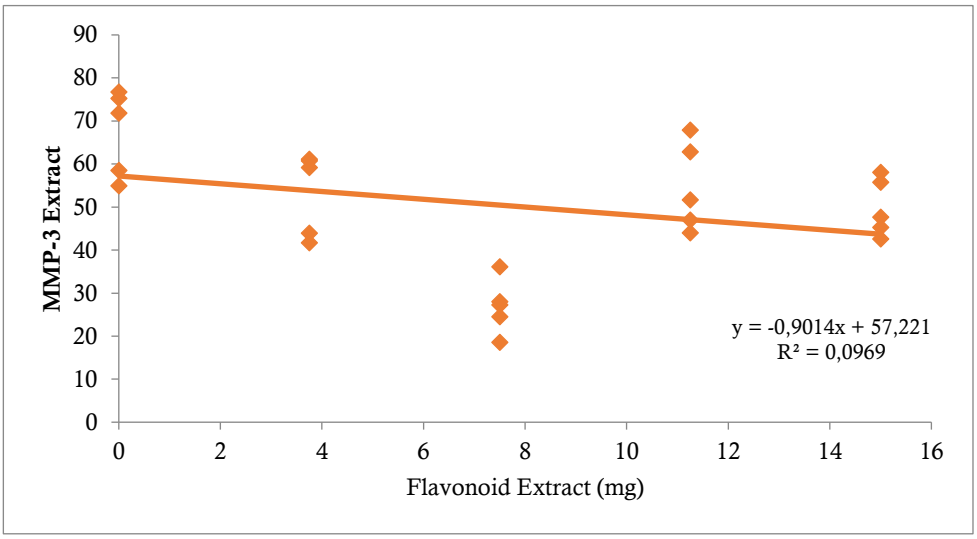

Fig. 2. Scatter Plot: Effect of Flavonoid Extract on MMP-3 Expression by Polynomial Regression

groups, where both treatment groups had letter notations different from the K+ group. This showed that giving flavonoid extracts from the *Phaleria macrocarpa* group P2 and P4 was able to reduce MMP-3 expression significantly. The positive control group (K+) had the highest average MMP-7 expression of 54.13 ± 4.08, statistically significantly different from the negative control group (K-). This is shown from the average value of ± elementary school in groups K- and K+, which contain different letters. Administration of flavonoid extracts from *Phaleria macrocarpa* fruit at all dose levels resulted in lower MMP-7 expression than in the K+ group.

Based on the results of the MMP-1 Expression regression analysis, a regression coefficient of -0.2832 was obtained with a p-value of 0.715. The coefficient of determination (R-square) of 0.59% shows that the diversity of data explained by the effect of giving Flavonoid Extract from the *Phaleria macrocarpa* fruit on increasing MMP-1 Expression is only 0.59%, while MMP-3 expression is only 9.69%. Very low R-square values indicate that linear models cannot explain the influence of flavonoid extract from *Phaleria macrocarpa* against MMP-1 and MMP-3 expression accurately. This happens because as the dose of Flavonoid Extract from *Phaleria macrocarpa* fruit increases, at certain doses, it has an impact on increasing and decreasing MMP-1 and MMP-3 Expression. Thus, a more complex regression model, namely polynomial regression, is needed.

Based on the results of the polynomial regression analysis above (Fig. 1), the R-square value is 0.7793 or 77.93%. Administrating flavonoid extracts was able to affect changes in MMP-1 expression by 77.93%. The remaining 22.07% was explained by other factors not

involved in the study. Based on the results of the polynomial regression analysis above (Fig. 2), the R-square value is 0.7384 or 73.84%. Administrating flavonoid extracts was able to affect changes in MMP-3 expression by 73.84%. The remaining 26.16% was explained by other factors not involved in the study.

Based on the results of MMP-7 Expression regression analysis, a regression coefficient of -3.289 was obtained with a p-value of 0.000. The coefficient of determination (R-square) of 91.67% shows that the diversity of data explained by the effect of giving flavonoid extract on increasing MMP-7 Expression is 91.67%. Very high R-square values indicate that linear models explain the influence of flavonoid extract against MMP-7 Expression very well and accurately. The R-square value of 91.67% contains the understanding that the effect of giving flavonoid extract can affect the decrease in MMP-7 expression by 91.67%. The remaining 8.37% was explained by other factors not involved in the study. The regression coefficient of -3,289 can be interpreted as the addition of flavonoid extract by 1 mg can reduce MMP-7 expression by 3,289.

DISCUSSION

Endometriosis, in which there is endometrial tissue outside of the uterus, is a benign gynecological condition. However, it can be aggressive. The ectopic endometrium shows proliferation, implantation, and angiogenesis and the possibility of surviving in different environments. The pathology of endometriosis is complicated and of multi-factor origin. Until now, the pathophysiology and etiology of endometriosis have not been widely known, although several theories have been revealed [5].

The development of endometriosis lesions involves immunosuppression factors, abnormal communication between the peritoneum and ectopic endometrium, and exosome activation[7]. There is a change in phenotype in peritoneal mesothelial cells in the form of ectopic endometrial cells to attach and attack the target, resulting in damage to the peritoneal pathway signal. Peritoneal mesothelial cells will release proinflammatory cytokines and growth factors (growth factors) into a stimulus for angiogenesis and endometriosis cell proliferation.

Previous studies say endometriosis is a multi-factor collection including immune, genetic, and hormonal environment characterized by abnormal expression of inflammatory factors. An important step in the development of endometriosis is the relationship between inflammation and activation of the aromatase gene in the endometrium, followed by local estrogen production in the endometrium [16]. MMP is a proteolytic enzyme that has an important role in the remodeling of the extracellular matrix in normal tissues but also contributes to pathologies such as tumor invasion [11]. This study aims to determine the effect of giving flavonoid extracts in various doses, namely 3.75 mg/day, 7.5 mg/day, 11.25 mg/day, and 15 mg/day, on reducing the index of cell apoptosis in mice model endometriosis [16].

Based on the results of analysis using ANOVA, a p-value of 0.000 was obtained, smaller than $\alpha = 0.05$ ($p<0.05$). So from this test, it can be concluded that there is a significant effect of giving flavonoid extracts on MMP-1, MMP-3, and MMP-7 expression. Post-hoc Tukey analysis shows a significant difference in the expression of these three metalloproteinases in each group, and it is also in line with regression analysis of each group and MMP expression. This is in line with research conducted by Sutrisno *et al.* (2018), where genistein, one of whose products is an isoflavone, can modulate estrogen receptors and suppress angiogenesis and inflammation in the murine model of peritoneal endometriosis. In addition, research conducted by Zahra *et al*. (2022) shows that isoflavones can prevent hyperplasia because of their effects on cell proliferation, apoptosis, and progesterone receptors.

This is also in accordance with a review article conducted by Balan *et al*. (2021), where they carried out studies on the pharmaceutical effects of medicinal plants and phytochemicals on endometriosis. The medicinal herb and its bioactive compounds exhibit anti-angiogenic and antioxidant effects that exert beneficial effects on endometriosis management. Bartimoro *et al*. (2021) also mentioned that phytoestrogens have many beneficial characteristics, such as anti-proliferative, anti-angiogenic, anti-inflammatory, pro-apoptotic, and anti-oxidant properties, which may make them viable alternatives in the future for endometriosis control and prevention.

CONCLUSION

Biocomponents of flavonoids from the *Phaleria macrocarpa* fruit extract were able to reduce the expression of MMP-1, MMP-3, and MMP-7 (proinflammatory factor) in mice model endometriosis. There is a correlation between the administration of flavonoids of Phaleria macrocarpa fruit extract and the expression of proinflammatory factors (MMP-1, MMP-3, and MMP-7).

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CONFLICT OF INTEREST

There is no conflict of interest in this research.

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