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Original Research Article (Experimental)

Phytochemical characteristics from *Phaleria macrocarpa* and its inhibitory activity on the peritoneal damage of endometriosis

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ABSTRACT

Background: Endometriois represents a gynecological disease that still becomes an issue in community. *Phaleria macrocarpa* is a plant native to Indonesia that contains an antioxidant substance, which may serve as apoptotic modulator and useful for angiogenesis.
Objective: This study aims to evaluate the effects of flavonoid isolates from *P. macrocarpa* (PM) on the development of granulomas, apoptosis, proliferation, and angiogenesis of the disease.
Material and methods: Total thirty mice (*Mus musculus*) were categorized into six groups, including the normal group (without any treatment), EMT (endometriosis) group, and EMT group treated with PM flavonoid isolates. Identification of the active compounds of *P. macrocarpa* was done using LC-HRMS. Measurement of granuloma scores and vascular density was done histologically. Apoptosis and proliferation analysis was performed by immunohistochemical techniques.
Results: There was an increase in granulomas, proliferation, and apoptosis in the peritoneal tissues of the endometriosis model. This change can be normalized by extract of *P. macrocarpa*.
Conclusion: We concluded that the flavonoid isolates from *P. macrocarpa* can suppress the growth of endometriosis lesions through normalization of proliferation and apoptosis. Thus, the *P. macrocarpa* flavonoid can be used as an alternative to inhibit the development of endometriosis.

1. Introduction

Endometriosis is a hormone-dependent gynecologic disease, with clinical manifestations of adhesion and progressive growth of endometrial cells in organs other than the uterus [1]. This disease affects a large portion of the population. Half of the patients with endometriosis display clinical symptoms of pelvic pain and infertility, thereby reducing the quality of life of women [2,3]. Until now, pathophysiology and etiology have not been known yet, although several theories have been revealed [4]. Initially, endometriosis occured due to retrograde menstruation, and now endometriosis is believed to occur due to chronic inflammation [5].

The development of endometriosis lesions involves immunosuppression factors, abnormal communication between the peritoneum and ectopic endometrium, and activation of exosomes [6]. There is a phenotype change in the peritoneal mesothelial cells in the form of ectopic endometrial cells to attach and invade the target [7], due to damage to the peritoneal pathway signals [8]. Besides, peritoneal mesothelial cells will release proinflammatory cytokines and growth factors as a stimulus for angiogenesis and proliferation [9].

Endometriosis lesions in the peritoneum are initially vascularized by blood vessels in the peritoneum. Also, interactions occur between mesothelial cells (dominant cells) and lesions [10]. Angiogenesis is controlled by VEGF and its receptors early in the development of endometriosis. Angiogenesis indicates the development of vascularity and cell proliferation behavior [11,12]. Previous studies have concluded that there was a disbalance between pro-angiogenic factors and antiangiogenic factors in endometriosis [6,13]. Nevertheless, some studies proved that angiogenesis in endometriosis did not involve changes in VEGF levels [14,15].

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Apoptosis is programmed cell death. External signals and autonomic genetic programs determine the course of apoptosis. Endometriosis has unique proliferation and apoptosis characteristics, in the form of increased cell proliferation and aggressiveness, apoptotic defects, supported by inflammatory conditions in the microenvironment [16–18]. Previous studies have shown a decrease in the apoptotic index in the stromal and glandular endometriosis patients compared with the controls [19,20]. This decrease is accompanied by changes in the profile of pro-apoptotic and anti-apoptotic proteins, as well as protein-related genes [21,22].

Phaleria macrocarpa is a plant native to Indonesia that is useful as food and medicine. This plant contains tannins, terpenoids, alkaloids, and flavonoids [23]. Previous studies have shown that standardized extracts of *P. macrocarpa* are antiproliferative and antiapoptotic [24,25]. An ethanol extract of *P. macrocarpa* triggers apoptosis of colon cancer cells [26]. A study on endometrial cells RL95-2 proved that the bioactive fraction of *P. macrocarpa* is antiangiogenic and pro-apoptotic [27]. As far as we know, there has been no study till date applying flavonoid isolates from *P. macrocarpa* for the treatment of endometriosis. Therefore, this study aims to evaluate the effects of flavonoid isolates from *P. macrocarpa* on the development of granulomas, apoptosis, proliferation, and angiogenesis of the disease.

2. Materials and methods

2.1. Animals

This study used female mice (*Mus musculus*), 12 weeks old, body weight 20–30 g in healthy condition. Totally thirty mice were randomly grouped into six groups, including a normal (control) group (n = 5); EMT (endometriosis) group (n = 5); EMT group received *P. macrocarpa* extract at a dose of 3.75 mg/day (n = 5); EMT group received *P. macrocarpa* extract (7.5 mg/day); EMT group received *P. macrocarpa* extract (11.25 mg/day); and EMT group received *P. macrocarpa* extract (15 mg/day). Flavonoid isolates were administered orally by gavage for 14 days. This dosage was determined based on previous studies [28] and has been proved as safe dosage [29].

2.2. Endometriosis model

Modeling of endometriosis mice was carried out according to previous study [30]. Characterization and diagnosis of endometriosis mice were made by examining the expression of estrogen receptors a and b in the peritoneal tissues [31].

2.3. Preparation of extracts

In this study the whole plant of *Phaleria macrocarpa* Boerl was obtained from Kota Langa, Aceh Province, Indonesia. It was verified by the curator in the Department of Biology, Faculty Mathematics and Natural Science, Syiah Kuala Univeristy, Banda Aceh, Indonesia under a voucher specimen number of B/430/UNI1.1.8.4/TA.00.01/2020. Two thousand and five hundred grams of *P. macrocarpa* flour were soaked in 30 L of 96% ethanol, then stirred (±30 min) until well mixed. The mixture was then allowed to stand for five nights until it settled. Next, filtering was done with a Buncher funnel to obtain the filtrates.

2.4. Separation of flavonoid

Separation of flavonoid compounds was carried out in order to have n-hexane and n-butanol partition. The ethanol extract was dissolved in n-hexane (1 L). After the precipitate was obtained, the n-hexane solution was removed, and the ethanol precipitate was evaporated at 45 °C. The separation was continued with n-butanol. The ethanol solution was mixed with n-butanol (centrifuged at 3000 RPM for 10 min). The supernatant was then taken and evaporated at 60 °C to obtain a concentrated flavonoid solution.

2.5. LC-HRMS

The extracted sample was diluted according to the solvent (polar). Dilution was done by looking at the thickness of the sample (not too thick and not too thin) with the final volume of 1300 uL. The sample was vortexed for 1 min and then spun down for 2 min. The supernatant was filtered using a 0.22 um syringe filter and put into vials. Samples in the vial were put into the Autosampler and then injected into the LC-HRMS.

The analysis was carried out by HPLC (Thermo Scientific Dionex Ultimate 3000 RSLC Nano with microflow meter). A 0.1% Formic acid in water (A) or Acetonitrile (B). The analysis used Hypersil GOLD AQ 50 × 1 mm x 1.9 u particle size with 40 uL/min flow. The running time was 30 min with temperature 30 °C in column oven.

2.6. Peritoneal isolation

Peritoneal isolation was performed according to the procedure in a previous study [30]. Peritoneal samples were stored at −80 °C before biomarker analysis was made.

2.7. Granuloma scoring

Granuloma score analysis was performed to determine the degree of peritoneal damage. The analysis was carried out based on modifications to the previous method [32]. A score of 0 if there is no granulation. A score of 2 if there is a granulation mass. A score of 4 if found granulation mass with an abscess. A score of 6 when a granulation mass is found with abscess, necrosis, and muscle tissue. A score of 6 for depicting granulation masses with abscesses, necrosis, muscle tissue, and fibrosis. The examination uses a Nikon H600L light microscope and its software.

2.8. Analysis of apoptosis

Measurement of apoptosis in peritoneal tissue was done immunohistochemically using the TUNEL technique, according previous study [33]. Apoptosis was determined by calculating the apoptosis index, which is the percentage of the apoptosis of the total cells per field with a 400× magnification light microscope. Observations were expressed in% (percent) [34].

2.9. Analysis of KI65 expression

Ki67 expression was assessed semiquantitatively according to a protocol in the prior method [34]. The index was determined by multiplying the percentage score of reactive cells with the color intensity score. The data were observed in 5 fields of view (x400 magnification), which have been previously confirmed at x1000 magnification. The entire examinations used a Nikon Eclipse Ci light microscope equipped with an Optilab Advanced digital camera and Nikkon Image System.

2.10. Ethics

Polytechnic of Health-Ministry of Health, Aceh Besar, Aceh, Indonesia (No. LB. 02.01/4583/2017).

Table 1
Composition of phytocomponent in the *Phaleria macrocarpa* flavonoid isolates.

Peak	Retention time	Name of compound	Formula	Molecular weight
1	1.41	Eriodictyol	$C_{15}H_{12}O_6$	288.0624
2	0.786	Glycitin	$C_{22}H_{22}O_{10}$	464.1305
3	1.374	5-O-methylgenistein	$C_{16}H_{12}O_5$	284.0676
4	0.751	(+)-catechin 7-O-beta-D-xyloside	$C_{20}H_{22}O_{11}$	422.1202
5	0.804	(-)-8-Prenylnaringenin	$C_{20}H_{20}O_5$	340.1301
6	0.801	(±)-Naringenin	$C_{15}H_{12}O_5$	272.0675

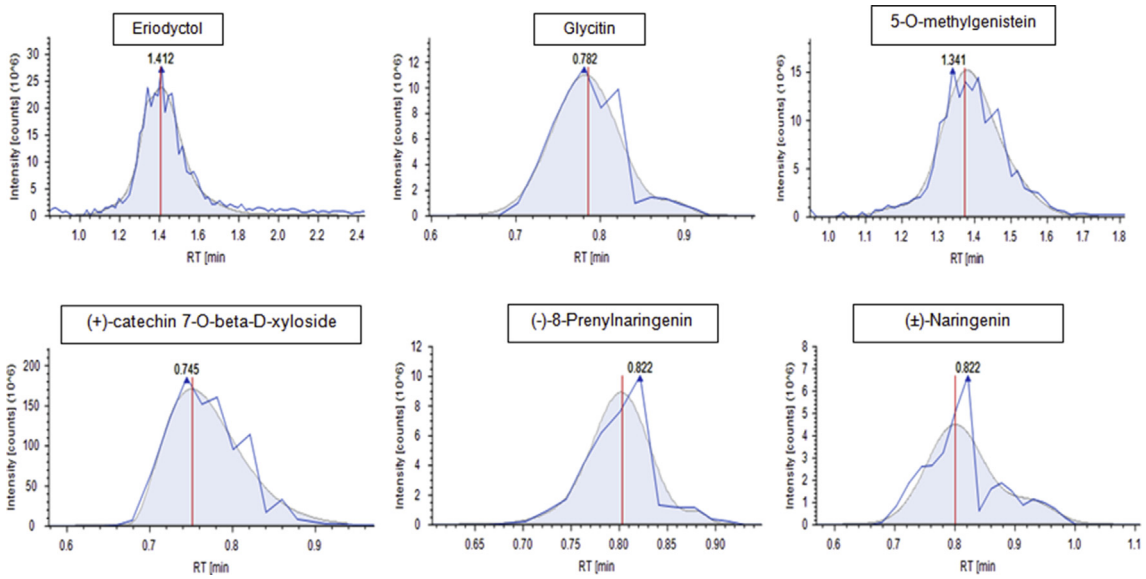

Fig. 1. Single chromatogram of active compounds from *Phaleria macrocarpa*.

2.11. Statistical analysis

Data are presented as mean ± SD, and the ANOVA test analyzed differences between treatment groups. The analysis was carried out with the SPSS 23.0 statistical package for Windows program. The probability value ($p < 0.05$) was stated to be significantly different.

3. Results

P. macrocarpa flavonoid extract contains 1) eriodictyol; 2) glycitin; 3) 5-O-methylgenistein; 4) (+)-catechin 7-O-beta-D-xyloside; 5) (-)-8-prenylnaringenin; and 6) (±)-naringenin, as shown in Table 1 and Fig. 1.

Granuloma tissue from various groups can be seen in Fig. 2. In the EMT group, there was a thick granuloma tissue compared with the control group. This granuloma tissue can be restored as in healthy group at the extract dose of 7.5 mg/day and 15 mg/day.

Table 2 shows the average granuloma scores between groups. There was an increase in granuloma scores in all the EMT groups compared with the healthy group ($p < 0.05$). The second-lowest and highest doses of flavonoid isolate significantly reduced granuloma peritoneum scores compared with the EMT group, achieving a value similar to the healthy group ($p > 0.05$).

Fig. 3 displays the proliferation markers from various groups. KI65 expression in the peritoneal tissue was significantly higher in the EMT group than in the control group ($p > 0.05$). This increase can be restored by all extract doses, reaching a value in the control group.

The apoptotic index of various groups can be seen in Fig. 4. The apoptotic index in the EMT group was significantly higher than the control group ($p < 0.05$). All extract doses can significantly reduced apoptosis compared with the EMT group ($p < 0.05$) but have not been able to reach normal values.

Peritoneal vascular density from various study groups can be seen in Table 3. Peritoneal vascular density did not differ significantly between treatment groups ($p > 0.05$).

4. Discussion

In this study, granuloma formation is a marker of peritoneal damage. We proved that peritoneal damage was quantitatively and qualitatively more significant in the endometriosis group compared

Table 2
Scoring of granuloma in various groups.

Marker	Control	EMT	EMT + PM_1	EMT + PM_2	EMT + PM_3	EMT + PM_4
Granuloma	0.4000 ± 0.8944	4.8000 ± 1.788[a]	2.000 ± 2.000	0.4000 ± 0.8944[b]	1.6000 ± 2.1908	0.8000 ± 1.0954[b]

Note: Value was presented as mean ± standard of deviation; EMT: endometriosis; PM_1: Flavonoid isolated of *Phaleria macrocarpa* at dose of 3.75 mg/day; PM_2: Flavonoid isolated of *Phaleria macrocarpa* at dose of 7.50 mg/day; PM_3: Flavonoid isolated of *Phaleria macrocarpa* at dose of 11.25 mg/day; PM_4: Flavonoid isolated of *Phaleria macrocarpa* at dose of 15.00 mg/day. [a]: $p < 0.05$ in comparison with control group; [b]: $p < 0.05$ in comparison with endometriosis group.

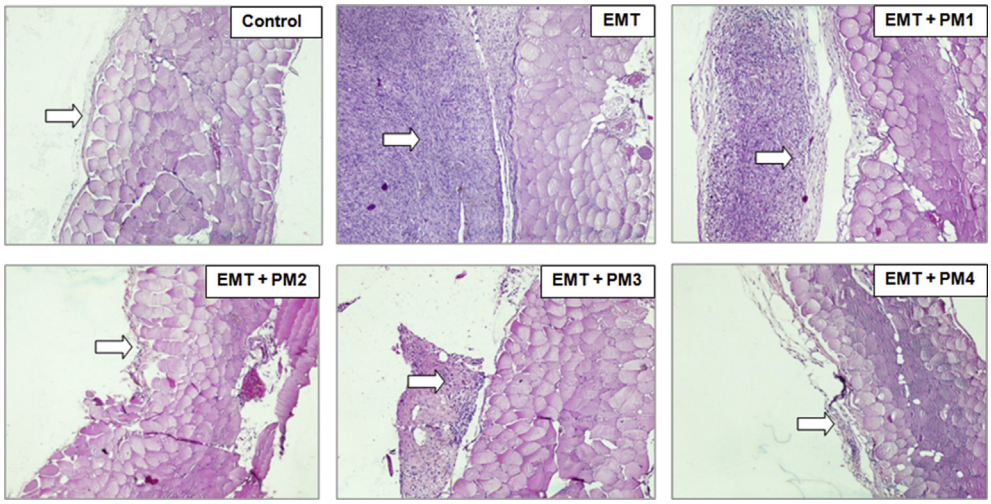

Fig. 2. The histology of granulomas from various groups. In the endometriosis group, granuloma tissue (white arrow) was found to be very dominant compared with the control. Extracts of this plant can restore granuloma tissue to achieve histology comparable to control at the second and fourth doses. Note: hematoxylin-eosin staining, magnification 100x; control; endometriosis group (EMT); endometriosis group which was given an extract at dose of 3.75 mg/day (EMT + PM1); endometriosis group which was given an extract at dose of 7.5 mg/day (EMT + PM2); endometriosis group which was given an extract at a dose of 11.25 mg/day (EMT + PM3); and the endometriosis group which was given an extract at dose of 15 mg/day (EMT + PM4).

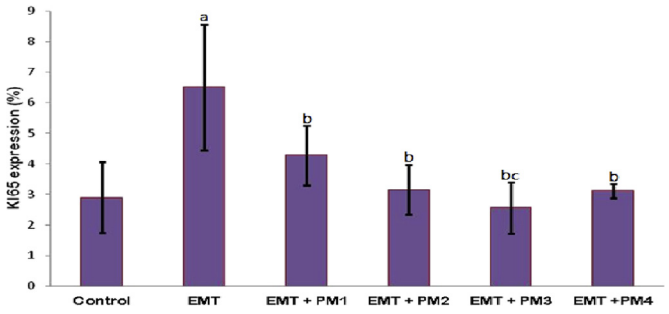

Fig. 3. Ki67 expression as proliferation marker in various groups. Data is displayed as mean ± standard deviation. Note: EMT = endometriosis; PM = *Phaleria macrocarpa*. a: significantly different compared with the control group ($p < 0.05$); b: significantly different compared with the EMT group ($p < 0.05$); c: significantly different than the EMT + PM1 group ($p < 0.05$).

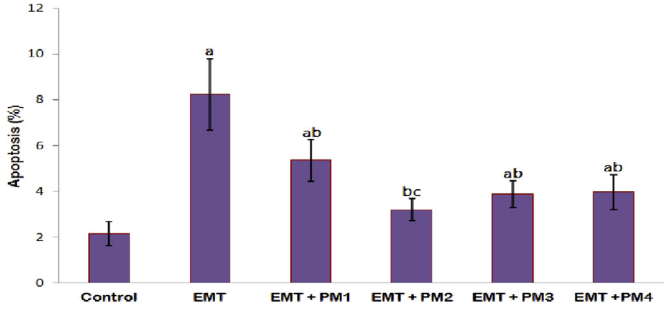

Fig. 4. Apoptosis index in the control and treatment groups. Data are expressed as mean ± standard deviation. Note: EMT (endometriosis); PM (*Phaleria macrocarpa*). a: significantly different compared with the control group ($p < 0.05$); b: significantly different compared with the EMT group ($p < 0.05$); c: significantly different than the EMT + PM1 group ($p < 0.05$).

with controls. The mechanism of granuloma formation was based on cell growth and angiogenesis. Our study proved increases in cell proliferation and apoptosis in endometriosis compared with domestic mice. Our study extends previous findings that apoptotic expression varies, depending on the location of endometriosis [20,22]. Nonetheless, our study is not consistent with previous findings which stated that in endometriosis animal models, there were no changes in cell proliferation [35].

In this study, there were significantly higher granuloma scores in endometriosis compared with the control group (Table 2). This finding indicates that there are an implant invasion and lesion development in the context of the development of endometriosis in the peritoneum. The development of endometriosis lesions includes three stages, namely red lesion (non-opaque or opaque), black, or white lesion [36]. We found that the mean granuloma score was four, as abscesses accompanied granulation lesions. These lesions included opaque red lesions to black/white lesions. This was supported by the behavior of cell proliferation and apoptosis that is not significant between various groups. These findings are consistent with the previous studies that opaque red lesions leading to black/white lesions had a low cell proliferation index. Vascular density values strengthen this finding that the mean vascular density did not differ significantly between study groups. Vascular density in endometriosis at the beginning of the low-value phase increased in the middle of the phase and eventually decreased again [37]. This result extends the previous findings that there is abnormal neoangiogenesis in endometriosis lesions [38].

In the study, extracts of 7.5 mg/day and 15 mg/day can suppress the growth of granulomas. This suppression mechanism is at least through the antiproliferative and antiapoptotic effects, which were proven in this study. Various active compounds from the extract are indeed involved in this mechanism. This study extends previous findings that *P. macrocarpa* extract can trigger apoptosis of endometrial cells [28].

Table 3
Peritoneal vascular density in various groups.

Marker	Control	EMT	EMT + PM_1	EMT + PM_2	EMT + PM_3	EMT + PM_4
Vasculer density (mm)	0.0820 ± 0.0130	0.1420 ± 0.2567	0.0680 ± 0.0408	0.0500 ± 0.0070	0.0540 ± 0.0089	0.0520 ± 0.0164

Note: Value was presented as mean ± standard of deviation; EMT: endometriosis; PM_1: Flavonoid isolated of *Phaleria macrocarpa* at dose of 3.75 mg/day; PM_2: Flavonoid isolated of *Phaleria macrocarpa* at dose of 7.50 mg/day; PM_3: Flavonoid isolated of *Phaleria macrocarpa* at dose of 11.25 mg/day; PM_4: Flavonoid isolated of *Phaleria macrocarpa* at dose of 15.00 mg/day; mm: millimeter.

Previous studies have shown that naringenin can inhibit endometrial cell proliferation from endometriosis lesions [39]. We proved that *P. macrocarpa* contains (±) -naringenin and its derivatives, (−) - 8-prenylnaringenin can inhibit proliferation and apoptosis, which can also suppress the development of peritoneal endometriosis.

5. Conclusion

The results of our study concluded that the flavonoid isolates from *P. macrocarpa* could suppress the growth of endometriosis lesions through normalization of proliferation and apoptosis. Thus, the *P. macrocarpa* flavonoid can be used as an alternative to inhibit the development of endometriosis.

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Conflict of interest

None.

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