RESEARCH ARTICLE

Phaleria macrocarpa Fruit Ethanol Extract Reduces MDA and TNF-α, as well as Maintains Caspase-3 Levels of Endometriosis-induced Mice

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Abstract

BACKGROUND: Endometriosis, which often causes infertility, is characterized by a complex interplay between oxidative stress, inflammatory response, and apoptosis. Currently, the most effective therapy used for endometriosis is hormonal treatments, however it has many common side effects. Therefore, it is necessary to explore alternative treatments for endometriosis, including herbal ingredients. *Phaleria macrocarpa* fruit contains flavonoids with strong antioxidant and anti-inflammatory properties which have potency to improve fertility in endometriosis cases. However, assessments of whether *P. macrocarpa* might have any effect on oxidative stress, inflammatory response, and apoptosis in relation to endometriosis are still limited. This study was conducted to identify the effect of *P. macrocarpa* fruit ethanol extract (PMFEE) in improving infertility by assessing oxidative stress, inflammatory response, and apoptosis parameters in endometriosis mice model.

METHODS: Female *Balb*/C mice were endometriosis-induced, and treated either with 6.5 µg/day Letrozole, 7.5 mg/kgBW/day PMFEE, or 15 mg/kgBW/day PMFEE for 14 days. Heatlhy mice were also included as control. Mice were necropsied, then the uterus and fat tissue around the uterus was collected. Malondialdehyde (MDA) as oxidative stress marker, tumor necrosis factor (TNF)-α as inflammatory marker, and Caspase-3 as apoptosis marker were assessed using enzyme-linked immunosorbent assay (ELISA).

RESULTS: In uterus, MDA and TNF-α levels were significantly different among all groups ($p<0.05$). PMFEE both at 7.5 and 15 mg/kgBW/day lowered MDA and TNF-α compared to the healthy mice and mice receiving Letrozol. Meanwhile, Caspase-3 levels were quite indifferent among groups, showing that PMFEE was able to maintain the Caspase-3 levels in the endometriosis-induced mice.

CONCLUSION: PMFEE reduces the MDA and TNF-α, also maintains Caspase-3 levels in the uterus of endometriosis-induced mice, suggesting it might have potential to improve fertility in endometriosis mice.

KEYWORDS: *Phaleria macrocarpa*, endometriosis, infertility, MDA, TNF-α, Caspase-3

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Introduction

Endometriosis is defined by the chronic presence of endometrial tissue proliferation outside the uterine cavity. (1) Approximately one in every ten reproductive age women between 12–52 years old encounters endometriosis. (2-3) Some studies showed that endometriosis is associated with inherited genetic traits, but the exact ethiopathogenesis remains unknown.(4) Women with endometriosis often experience infertility and pain (5), which can be attributed to the inflammatory process (6). The increasing levels of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-8 are known to reduce ovarian responsiveness (7), since they are responsible for promoting adhesions within the pelvic region of women with endometriosis (8). High TNF-α level is reported to contributes to endometriosis infertility by failing the implantation.(9)

The overexpression of both proinflammatory cytokines and reactive oxygen species (ROS) in endometriosis promotes apoptosis, which further leads to various organ impairment.(10) In endometriosis, the elevated levels of malondialdehyde (MDA), which is known as a stable and accurate biomarker of oxidative stress (11-13), reflect increased oxidative stress and lipid peroxidation, which contribute to cellular damage and a proinflammatory peritoneal environment (14). Oxidative stress promotes the proliferation and attachment of endometrial cells and stimulates angiogenesis to support the growth of endometrial tissue in the peritoneal cavity thus inducing endometriosis and contributing to infertility.(15) This oxidative imbalance is closely associated with dysregulation of Caspase-3, a key executor of apoptosis (16), leading to reduced programmed cell death of ectopic endometrial cells. As protein representative of cell apoptosis, Caspase-3 is usually used as apoptosis biomarker for endometriosis severity. (17,18) This suggests that the interplay between increased proinflammatory cytokines such as TNF-α, elevated MDA levels, and impaired Caspase-3 activity might promotes the survival and persistence of endometriotic lesions.

Current available treatments that are widely and most effective used for endometriosis therapy is hormonal treatments which are based on the pathogenic mechanisms involved in the disease. However, this treatments are not with no side effects. The most common side effects occur do to these therapies including hot flashes, sleeping problems, headache, depression, joint and muscle stiffness, bone loss, or vaginal dryness. Therefore, the use of drugs from herbal therapy, such as epigallocatechin gallate (EGCG), curcumin, puerarin, and resveratrol, needs to be further explored as alternative choices for endometriosis therapy.(19)

One of native herbal plant from Papua, Indonesia, *Phaleria macrocarpa*, which is locally known as Mahkota Dewa, is reported to contains flavonoids, alkaloids, terpenoids and steroids (20), as well as inflammatory properties potential due to the terpenoids, saponins, tannins, flavonoids, and phenols in the fruit (21). The strong antioxidant and anti-inflammatory properties contained in this fruit might be potential to improve fertility in endometriosis cases. Recent studies showed the benefit of *P. macrocarpa* extract in managing premenstrual syndrome, and dysmenorrhea (22), relieving the pain associated with endometriosis and adenomyosis, which persists for two treatment cycles. However, assessments of whether *P. macrocarpa* might have any effect on oxidative stress, inflammatory response, and apoptosis in relation to endometriosis are still limited. Therefore, this study was conducted to identify the effect of *P. macrocarpa* fruit ethanol extract (PMFEE) in improving infertility by assessing MDA as stress oxidative marker, TNF-α as pro-inflammatory marker, and Caspase-3 levels as apoptosis marker, in endometriosis mice model.

Methods

Preparation of PMFEE

Fruit of *P. macrocarpa* (Scheff.) Boerl was obtained from Hargobinangun Plantation, Pakem, Sleman, Yogyakarta and identified at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta (Identification No.21.22.3/UN1/FFA.2/S1/PT/2022, dated March 22, 2022). A total of 1 kg of *P. macrocarpa* fruit simplicia was macerated with 95% ethanol for 24 hours. The dregs were re-macerated with 95% ethanol for another 24 hours. The extracts from both maceration processes were combined and filtered, then concentrated using a rotary evaporator to obtain a thick crude extract. The concentrated extract was further dried in a water bath to remove residual solvent and weighed to determine the extraction yield. The crude extract was subsequently reconstituted in 0.5% carboxymethyl cellulose (CMC-Na) solution as a suspending agent to obtain the desired concentrations for administration. Crude extract for treatment was prepared daily with dose calculation based on the individual body weight of experimental mice, with treatment groups receiving either 7.5 or 15 mg/kgBW/day of PMFEE.

Animal Grouping

Twenty female *Balb*/C mice aged 1.5-2 months and weighed 20-30 gram were included in this study and each was put in a cage with a minimum height of 12 cm, good ventilation, no noise, proper lighting (12 hours of dark and light), room temperature (20-24°C), relative humidity of 45-65% before the study. The mice were then randomly assigned into 4 groups, with 5 mice in each groups: 1) Endometriosis-induced mice which were given the standard drug of 6.5 µg/day Letrozole; 2) Endometriosis-induced mice which were given 7.5 mg/kgBW/day PMFEE; 3) Endometriosis-induced mice which were given 15 mg/kgBW/day PMFEE; and 4) Healthy mice as the normal control.

Endometriosis Induction and Animal Treatment

Twelve hours before the induction, the endometriosis-induced mice were subjected to fasting. On day-0, fresh chocolate cyst slurry was given by intraperitoneal injection. Cyclosporine was then given by intraperitoneal injection over 14 days to induce immunodeficiency. On day-1 and day-5, estrogen was administered by intramuscular injection to induce endometriosis.

Fourteen days after the endometriosis induction, all endometriosis-induced mice received different treatment according to their group allocation for 15 consecutive days via oral administration using gastric gavage. Meanwhile, the healthy mice were not subjected to any induction nor treatment for whole study (Figure 1). After 15 days of treatment, all mice including the healthy mice, were necropsied with ketamine and the surgery was conducted by removing the uterus and peritoneal fat around the uterus. All procedures performed in this study was in accordance with the ethically approved study protocol.

Hematoxylin-eosin (H&E) and immunohistochemistry (IHC) Examination

To confirm whether the endometriosis induction was successful or not, the presence of endometrial-like glands accompanied by surrounding stromal tissue (stromatosis) in the uterus was assessed by anatomical pathology examination using H&E staining was performed according to previous publication.(23) The collected uterine samples were fixed in 10% neutral buffered formalin for 24–48 hours, trimmed and processed through graded alcohol dehydration, xylene

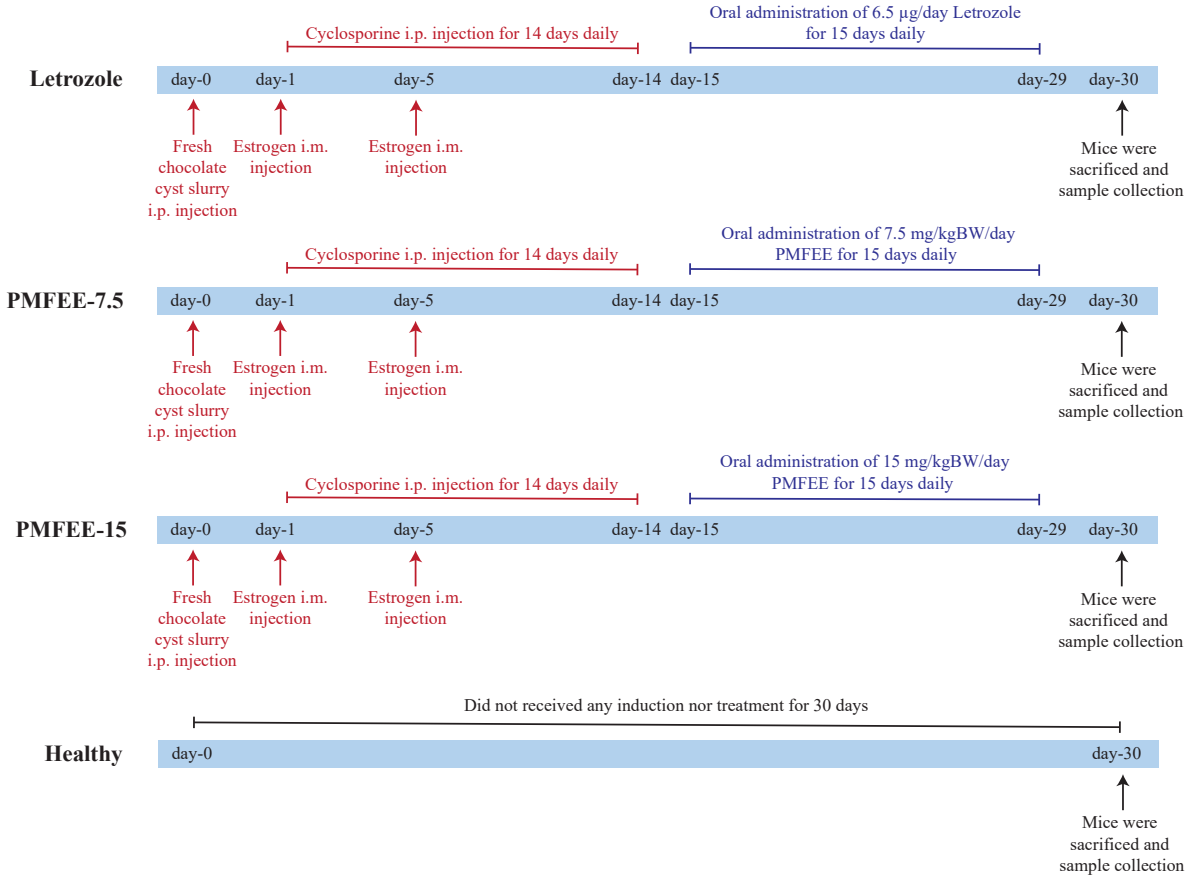

Figure 1. The study timeline and treatments of the experimental groups. The procedures written in red fonts are the endometriosis-induction, while the procedures written in blue fonts are the treatment according to the group allocation.

clearing, and paraffin embedding. The paraffin blocks were sectioned at 3–5 µm thickness and mounted onto glass slides. After dehydration and mounting, the slides were examined by an anatomical pathology specialist to evaluate histomorphological features, particularly the presence of endometrial glands and stromatosis as indicators of endometrial tissue characteristics.

Meanwhile for the fat tissue, IHC examination was performed to confirm the endometriosis. The sample paraffin-embedded tissue blocks were sectioned at 4 µm thickness and mounted on poly-L-lysine-coated slides. Sections were deparaffinized in xylene, rehydrated through graded alcohol, and treated with H_2O_2 for 15 minutes to block endogenous peroxidase activity. Antigen retrieval was performed using citrate buffer (pH 6) in a microwave for 2 × 5 minutes, then cooled for 15 minutes and rinsed. Non-specific binding was blocked using normal serum for 10 minutes, followed by incubation with anti-ER monoclonal antibody (Ventana SP-1 RTU; Ventana Medical Systems, Tucson, AZ, USA) overnight. Sections were then incubated with biotinylated secondary antibody and streptavidin-peroxidase. Immunoreactivity was visualized using diamino bezidine (DAB) chromogen for 5 minutes, followed by counterstaining with hematoxylin. The stained sections were subsequently evaluated by an anatomical pathology specialist specialist to identify both stromatosis and associated inflammatory cells. Stromatorsis was detected if dense and spindle-shaped cells closely surrounding gland-like structures were observed within the fat tissue. The detection of inflammatory cells was provided to dishtiguised stromatosis condition with other non-specific inflammatory features.

MDA, TNF-α, and Caspase 3 Enzyme-linked Immunosorbent Assay (ELISA)

The tissues collected from the uterus and peritoneal fat around the uterus were rinsed with ice-cold phosphate-buffered saline (PBS) (pH 7.4) to remove any remaining blood and weighed. The tissue was minced and homogenized in PBS (tissue weight (g):PBS volume (mL) = 1:9) in a homogenizer glass on ice. To further break down the cells, the suspension was sonicated with an ultrasonic cell disrupter. Subsequently, the homogenate was centrifuged at 10,000 RPM for 5 minutes, and the supernatant was collected for MDA, TNF-α, and Caspase 3 analysis with ELISA.

The procedures were performed according to the kit manual of Mouse Malondialdehyde ELISA Kit (Cat. No. BZ-08145260-EB; Bioenzy, Jakarta, Indonesia), Mouse Tumor Necrosis Factor α ELISA Kit (Cat. No. BZ-08147110-EB; Bioenzy), and Mouse Caspase 3 ELISA Kit (Cat. No. BZ-08143151-EB; Bioenzy). For each kit, 50 µL standard was added to the standard well. While 40 µL sample was added to the sample wells followed by 10 µL anti-MDA antibody. Fifty µL streptavidin-HRP was then added to standard and sample wells and mixed, before incubated for 60 minutes at 37°C. After incubation, the plate was washed multiple times with wash buffer to remove unbound components. Subsequently, 50 µL substrate solutions were added and the plate was incubated for 10 minutes at 37°C in the dark. The reaction was stopped by adding 50 µL stop solution, resulting in a color change from blue to yellow. The optical density (OD) of each well was immediately measured at 450 nm using a microplate reader. The obtained absorbance values were used to determine the concentration of the target analyte in the samples based on the standard curve.

Results

Endometriosis induction was successful, which is confirmed by the presence of stromatosis found in the uterus (Figure 2A) and fat tissue (Figure 2B) of the induced mice. Strong positive nuclear staining of estrogen receptor in the fat tissue demostrated endometrial stromal cells, hence confirming their endometrial origin. In this matter, stromatosis refers to the proliferation and accumulation of endometrial stromal cells in tissues other than uterus. In addition to the stromal component, infiltration of surrounding inflammatory cells (green arrow) was observed, distinguishing stromatosis from other non-specific inflammatory cells (Figure 2B). These findings indicate the successful establishment of an endometriosis model in mice.

Lower MDA Levels in Uterus After PMFEE Administration

In the uterus, there were significant difference of MDA levels among the 4 experimental groups (p=0.013). MDA levels of the healthy mice and the mice receiving Letrozole were relatively similar (1.72±0.14 and 1.81±0.15 ng/mL, respectively), showing that endometriosis-mice treated with standard drug might be able to reduce oxidative stress reflected by MDA level to almost normal level. The administration of PMFEE both in 7.5 and 15 mg/kgBW/day showed even lower MDA levels compared to the healthy mice and the mice receiving Letrozole, with mice receiving 15 mg/kgBW/day PMFEE showing the lowest MDA level (1.30±0.41 ng/mL) (Figure 3A).

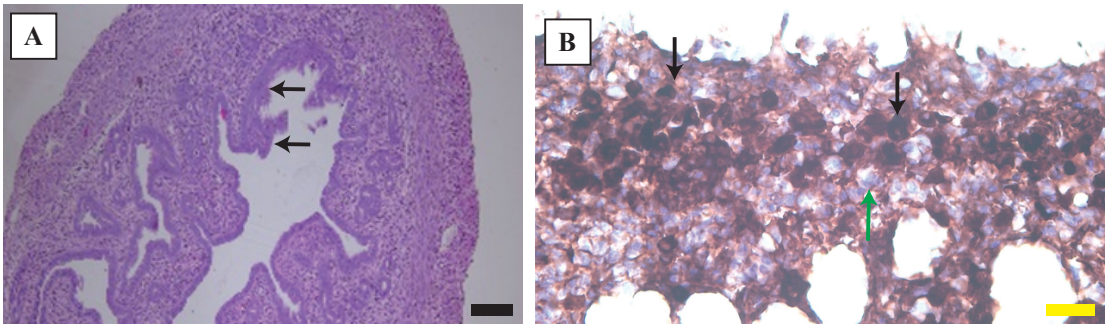

Figure 2. H&E and IHC results confirming the establishment of the endometriosis mice model. The endometriosis induction was confirmed by the presence of stromatosis in uterus by H&E staining (A) and in fat tissue by IHC examination (B). Black arrow: stromatosis (dark dots). Green arrow: inflammatory cells. Black bar: 20 µm; Yellow bar: 100 µm.

However, in the fat tissue, there was no significant difference of MDA levels observed among the study group (p=0.057), eventhough the mice receiving 7.5 mg/kgBW/day PMFEE demonstrated lowest level of MDA compared to other groups (2.06±0.30 ng/mL) (Figure 3B). Hence, the reduction of MDA as a marker of oxidative stress in the treatment groups suggesting that the treatment with PMFEE might attenuate oxidative stress more effectively in uterus compared to fat tissue.

Lower TNF-α Levels in Uterus After PMFEE Administration

Similar with MDA level, in uterus, there were significant difference of TNF-α levels among the 4 experimental groups (p=0.022). In the healthy mice and mice receiving Letrozole, the TNF-α levels were relatively similar (198.41±25.25 and 183.12±41.98 pg/mL, respectively), showing that Letrozole treatment was able to reduce TNF-α level of endometriosis-mice to normal level. The pro-inflammatory cytokines, TNF-α found to be even lower in both PMFEE administrated groups compared to the healthy and Letrozole-treated mice (Figure 4A). TNF-α levels reflect the activity of inflammatory cells such as macrophages and lymphocytes. In this study, both PMFEE-treated groups showed reduced TNF-α levels in uterus, indicating a suppression of inflammatory response after the PMFEE administration at both 7.5 and 15 mg/kgBW/day PMFEE.

Meanwhile, the in fat tissue, TNF-α levels ranged from 211.33±21.87 to 241.88±10.64 pg/mL and showed no significant difference among the experimental groups (p=0.391), with (Figure 4B), indicating a less pronounced anti-inflammatory effect in fat tissue. This may reflect persistent activation of local inflammatory cells within the peritoneal environment, known as a critical role in the maintenance and progression of endometriotic lesions. These results suggesting that PMFEE might have an anti-inflammatory effect specific to uterus.

PMFEE Administration Modulates Caspase-3 Expressions in Uterus

Caspase-3 is a key executor of apoptosis; however, as the non-cleaved Caspase-3 was measured in this study, the findings reflect overall expression rather than direct apoptotic activity. In the uterus, there was a significant

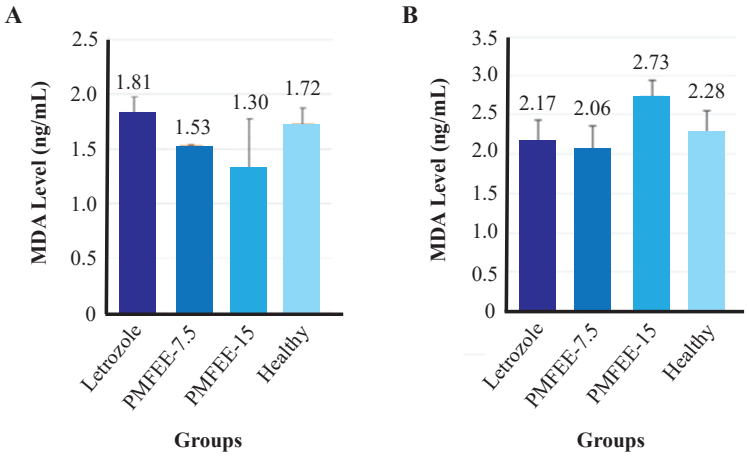

Figure 3. Difference of MDA levels between the study groups. A: MDA level in uterus; B: MDA level in fat. Data were shown as mean±standard deviation (SD). Letrozole: Endometriosis-induced mice treated with standard drug of 6.5 µg/day Letrozole; PMFEE-7.5: Endometriosis-induced mice treated with 7.5 mg/kgBW/day PMFEE; PMFEE-15 Endometriosis-induced mice treated with 15 mg/kgBW/day PMFEE; Healthy: Healthy mice.

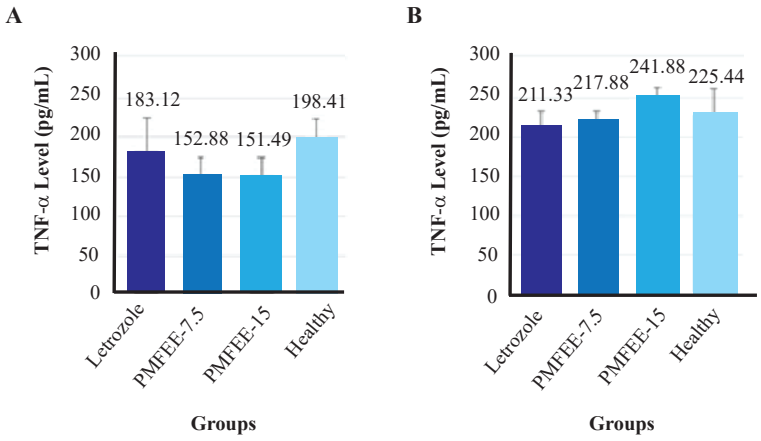

Figure 4. Difference of TNF-α levels between the study groups. A: TNF-α level in uterus; B: TNF-α level in fat. Data were shown as mean±SD. Letrozole: Endometriosis-induced mice treated with standard drug of 6.5 µg/day Letrozole; PMFEE-7.5: Endometriosis-induced mice treated with 7.5 mg/kgBW/day PMFEE; PMFEE-15 Endometriosis-induced mice treated with 15 mg/kgBW/day PMFEE; Healthy: Healthy mice.

difference among the study groups (p=0.040), eventhough in the healthy mice as well as in the 7.5 and 15 mg/kgBW/day PMFEE-treated mice, Caspase-3 level remained relatively similar (ranged from 1.48±0.20 to 1.94±0.28 ng/mL) (Figure 5A). The lower Caspase-3 levels observed in both PMFEE-treated groups could suggest that PMFEE help maintain or regulate cell death processes, potentially by promoting a more balanced Caspase-3 expression that supports controlled cell turnover without excessive inflammation or stress-induced overexpression.

In contrast, in the fat tissue, non-cleaved Caspase-3 expressions showed no statistically significant differences among the groups (p=0.080), and ranged almost similar from 2.12±0.19 to 2.77±0.30 ng/mL (Figure 5B). The relatively unchanged Caspase-3 levels might suggest that apoptotic activity was not markedly elevated, and therefore cell death may not be prominent or easily detectable under these conditions. In fat tissue, the inflammatory status was likely indifference between the treated endometriosis-induced mice and healthy mice, and as a result, both Caspase-3 expression and apoptosis-related changes were minimal and did not show substantial variation across groups.

Discussion

The present study demonstrates that administration of PMFEE exhibited a modulatory effect on oxidative stress and inflammation in a mice model of endometriosis, which is supported by the significant reduction of MDA and TNF-α levels in the uterus following the treatment of PMFEE. Some studies reported that oxidative stress which is reflected by serum MDA levels were elevated in mice with endometriosis compared to the healthy mice counterpart (24-26), showing that endometriosis-induction in mice followed with no treatment may cause elevated oxidative stress. Similarly, TNF-α expressions are also reported to be higher in endetriosis mice with no treatment compared to the healthy and other treated group.(27)

In current study, both treatment with 7.5 and 15 mg/kgBW/day PMFEE demonstrated lower level of MDA and TNF-α in uterus compared to healthy and Letrozole-treated endometriosis mice (Figure 3, Figure 4). This was in accordance with the results of previous study which found that 7.5 mg/day and 15 mg/day of *P. macrocarpa* extract

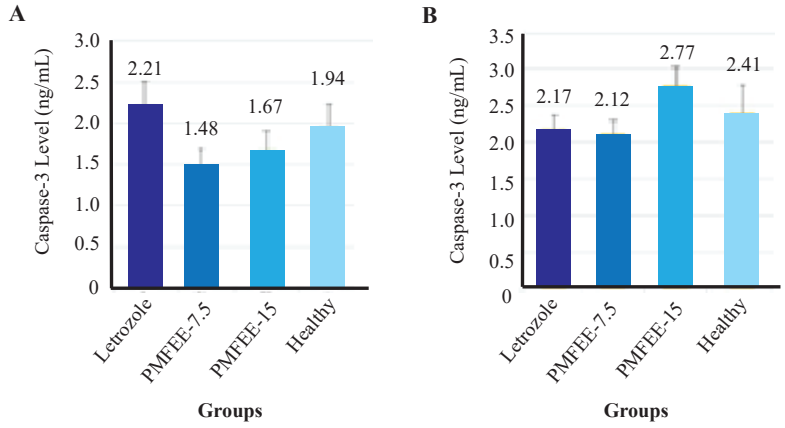

Figure 5. Difference of Caspase-3 levels between the study groups. A: Caspase-3 level in uterus; B: Caspase-3 level in fat. Data were shown as mean±SD. Letrozole: Endometriosis-induced mice treated with standard drug of 6.5 µg/day Letrozole; PMFEE-7.5: Endometriosis-induced mice treated with 7.5 mg/kgBW/day PMFEE; PMFEE-15 Endometriosis-induced mice treated with 15 mg/kgBW/day PMFEE; Healthy: Healthy mice.

suppresses the growth of granulomas and peritoneal damage in endometriosis lesions.(28) Other herbal ingredients, such as Kebar grass and green tea, are also shown to diminish endometriosis tissue implantation in mice due to their antioxidant property, thus decreasing the MDA level and TNF-α expression compared to the non-treated control group. (29,30) The flavonoid and phenolic content in *P. macrocarpa* fruit might be the reason for its strong antioxidant properties (31), since flavonoids and phenol prevent the generation of ROS, capture ROS directly, and increase the enzymes to capture ROS (32). In addition to antioxidants, flavonoids also have anti-inflammatory properties (33,34), strengthening the assumption that PMFEE may improve the fertility in the endometriosis mice model. However, its effect might be specific to uterus or within endometrial environment, since it does not show any significant results in fat. This disparity indicates a tissue-specific response to treatment, suggesting that the uterine environment may be more responsive to the extract compared to ectopic or adipose-associated tissues. The persistent inflammatory milieu within fat tissue, which is known to produce cytokines and support lesion survival, may limit the therapeutic effect of PMEE in such environment.

The modulation of Caspase-3 suggests its role in maintaining the balance of cell death in response to inflammation. In endometriosis, elevated TNF-α can stimulate apoptotic signaling as a compensatory mechanism to eliminate damaged or ectopic cells.(34) However, excessive or persistent inflammation may lead to dysregulated apoptosis. In current study, non-cleaved Caspase-3 expression were measured, so the level of Caspase-3 observed in this study might not necessarily equate to increased apoptosis. Caspase-3 expression is significantly decreased in ectopic and eutopic endometrial tissues of women with endometriosis compared to healthy controls, indicating impaired programmed cell death.(35)

In this study, the Caspase-3 levels measured in uterus only showed slight differences, while in fat was not significantly different among all groups. This suggests that Caspase-3 expression may be relatively stable and less affected by endometriosis induction or the treatments. In uterus, elevated Caspase-3 in the Letrozole-treated mice may reflect heightened cellular stress or inflammation rather than actual cell death, as endometriotic lesions often suppress apoptosis despite increased Caspase-3 expression. But the lower Caspase-3 level in PMFEE-treated groups may suggest that these extracts have the potential to maintain or regulate cell death processes, potentially by modulating inflammatory or apoptotic signaling pathways. In other words, the extracts might promote a more balanced Caspase-3 expression that supports controlled cell turnover without excessive inflammation or stress-induced overexpression.(36)

In accordance with previous study that reported the decrease of the levels of MDA, TNF-α, and Caspase-3 in the uterus after propolis administration (37), the current study also showed similar results after the administration of PMFEE both at 7.5 and 15 mg/kgBW/day. This suggest that interplay between oxidative stress, inflammation, and apoptosis in uterus tissue is central to the pathophysiology of endometriosis. And by modulating the respective pathway with ingredients that possess antioxidant, anti-inflammation, and anti-apoptotic properties may be potential to attenuate infertility due to endometriosis.

Overall, these findings suggest that PMFEE primarily exerts a protective effect by suppressing oxidative stress and inflammation rather than directly inducing apoptosis. This mechanism may contribute to the stabilization of cellular homeostasis in endometrial or uterus tissue. Nevertheless, the limited effect observed in fat tissue might highlights the complexity of the ectopic environment and suggests that additional or combination therapies may be required to effectively target this environment. And since non-cleaved Caspase-3 cannot distinguish between active and inactive forms, these results alone cannot confirm whether apoptosis is truly maintained or enhanced by PMFEE. Future studies incorporating active apoptotic markers, such as cleaved Caspase-3, are warranted to further elucidate the role of apoptosis in the therapeutic effects of this extract.

Conclusion

Oral administration of PMFEE reduces the MDA and TNF-α levels, also maintains Caspase-3 levels of endometriosis-induced mice. Especially in uterus, MDA and TNF-α levels are reduced more effectively after the administrarion of 7.5 mg/kgBW/day PMFEE. This suggests that PMFEE administration might be potential to improve fertility in endometriosis mice by modulating the oxidative stress, inflammatory, and apoptosis markers.

Acknowledgments

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Authors Contribution

IBB, S, and BP were involved in arranging concept and planning the research. IBB performed the data acquisition/collection. IBB and EPP calculated the experimental data and performed the analysis. IBB drafted the manuscript and designed the figures while BP and EPP aided in interpreting the results. BW, URB, MP, SL, ETW, SM prepared the final draft for the publication. All authors took part in giving critical revision of the manuscript.

Ethical Statement

The protocol of this study was approved by the Research Ethics Committee, Faculty of Medicine, Universitas Sebelas Maret, Surakarta, Indonesia (No. 95/UN27.06.11/KEP/EC/2022).

Conflict of Interest

The authors declare no conflict of interests

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