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Impacts of *Phyllanthus niruri* extract on biomarker levels, macrophage count, and lesion area in an endometriotic rat model

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Abstract

Endometriosis is a gynecological disorder characterized by chronic inflammation, anatomical changes, prolonged pain, and infertility. On the other hand, *Phyllanthus niruri* is recognized for its pharmacological effects, which might be beneficial in managing endometriosis. The aim of the study was to investigate the pharmacological effects of *P. niruri* as a potential therapy for endometriosis by using an animal model. An experimental laboratory study with randomized, controlled trial, pre-test, and post-test design using 40 female Wistar rats (*Rattus norvegicus*) was conducted at the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada, Yogyakarta, Indonesia, from February to June 2023. Endometriosis was induced in female Wistar rats by suturing a 0.5 cm^2 flap from the uterine horn to the peritoneal cavity. Changes in serum interleukin 1β (IL-1β), malondialdehyde (MDA), and matrix metalloproteinase 9 (MMP-9), before and after the treatment, were analyzed using enzyme-linked immunosorbent assay. Hematoxylin and eosin (HE) staining were used to evaluate lesion size and macrophage quantity. The results suggested that the *P. niruri* extract with a dose of 196 mg/200 g body weight (BW) could significantly attenuate serum IL-1β (p=0.004), MMP-9 (p=0.021), and MDA (p=0.021). Rats receiving the *P. niruri* extract (196 mg/200 g BW) had significantly higher macrophage counts (p=0.003), but similar lesion area (p=0.093) as compared with the negative control. In conclusion, *P. niruri* demonstrated promising therapeutic effects on endometriosis by modulating IL-1β, MDA, and MMP-9 levels, although the effect was not pronounced on macrophage counts and lesion area.

Keywords: Endometriosis, *Phyllanthus niruri*, inflammation marker, oxidative stress, rat model

Introduction

Endometriosis is a gynecological disorder defined as an inflammatory condition characterized by the presence of tissue lesions resembling the endometrium located outside the uterine cavity [1]. Endometriosis causes chronic inflammation, resulting in anatomical changes such as scar tissue formation and adhesions, prolonged pain, and infertility. The incidence of endometriosis is estimated to be 10–15% in women of reproductive age and it is identified in approximately 30% of women who seek medical attention due to infertility issues [2]. The definitive cause of endometriosis remains unknown despite various hypotheses that have been proposed. Chronic inflammation is one of the pathogenic factors in endometriosis. The inflammatory cascade leads to the upregulation of various inflammatory factors, including metalloproteinases,

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prostaglandins, chemokines, and cytokines. Macrophages are believed to have a significant role in immunosurveillance within the peritoneal microenvironment in endometriosis [3]. Macrophages are supposed to clear the peritoneal cavity of endometrial cells. However, in endometriosis, macrophages increase the proliferation of endometrial cells by secreting growth factors and cytokines [3]. Further, the macrophage activation consequently produces reactive oxygen species (ROS) in the peritoneal fluid, significantly contributing to the development of endometriosis [4].

The expression of IL-1β has been widely associated with endometriosis. IL-1β is a pro-inflammatory cytokine produced by macrophages that stimulate endometriosis cells to produce several cytokines and growth factors, which play crucial roles in adhesion, growth, invasion, inflammation, and angiogenesis in endometrial tissue [5]. Matrix metalloproteinases (MMPs) can degrade the extracellular matrix and are vital to endometrial adhesion and angiogenesis [6]. The formation of new blood vessels (angiogenesis) is necessary for endometriosis tissue to supply lesion formation or maintenance [7]. Among the various MMPs, Matrix Metalloproteinase-9 (MMP-9) has been particularly associated with the development of diseases characterized by abnormal angiogenesis, such as tumors and breast cancer [8]. Thus, targeting MMP-9 in endometriosis presents a promising therapeutic approach by potentially reducing the invasiveness of endometrial cells and limiting angiogenesis, thereby inhibiting the development and maintenance of endometriotic lesions.

In developing the therapeutic modality for this disease, researchers have investigated natural compounds from various sources with known pharmacological effects. In several studies, *Phyllanthus niruri* has been reported to have various pharmacological effects such as antiviral, antibacterial, antioxidant, anti-inflammatory, and immunomodulatory properties [9]. In a recent study, the immunomodulatory effects of *P. niruri* extract were evaluated, demonstrating its potential as a complementary therapy by normalizing immune responses such as natural killer cells, Th1 cells, and regulatory T-cells in a 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer mouse model [10]. In addition, *P. niruri* was found to help preserve near-normal kidney function and prevent histopathological changes by reducing oxidative stress, inflammation, fibrosis, and apoptosis [11]. This study is the first to investigate the pharmacological effects of *P. niruri* on endometriosis using a multi-pathway approach. The aim of this study was to evaluate the potential of *P. niruri* in treating endometriosis. It is worth noting that the assessment of endometriosis treatment cannot be approached through a single pathological pathway. Therefore, this study investigated the effects of *P. niruri* in terms of antioxidant, anti-inflammatory, immunomodulatory, and anti-angiogenesis activities as observed through relevant biomarkers and histopathological analysis.

Methods

Study design

This study is an experimental laboratory study with randomized, controlled trial, pre-test, and post-test design. The samples consisted of 40 female Wistar rats (*Rattus norvegicus*). The sample size was determined a priori using the G Power software application. After a 10-day acclimatization period, the rats were divided into five groups, each consisting of eight rats: normal group (normal rats without any additional treatment), PN 196 group (endometriosis group treated with *P. niruri* extract and 196 mg/200 g of body weight (BW)), PN 392 group (endometriosis group treated with *P. niruri* extract and 392 mg/200 g of BW), Dismeno group (endometriosis group treated with *Phaleria macrocarpa* with trade name Dismeno with of 5.4 mg/200 g BW, in accordance with the Indonesian herbal standard treatment for endometriosis), and negative control group (endometriosis group without any additional treatment). Laparotomy was performed on the PN 196, PN 392, Dismeno, and negative control groups to obtain an endometriosis model, followed by a 30-day observation period. On day 30, blood samples were collected for pre-treatment analysis of MDA, MMP-9, and IL-1β levels. Treatment using the plant extract was carried out on day 31 and 14 days onward. On day 46, the rats were euthanized, and tissue and blood samples were collected. The levels of MDA, MMP-9, and IL-1β were measured using enzyme-linked immunosorbent assay (ELISA) kits from the Bioassay Technology

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laboratory (BT lab) in China. The detailed ELISA kits' numbers were Rat IL-1β ELISA Kit (Cat. no E0119Ra 1), Rat MDA Assay Kit (Cat. no E0156Ra), and Rat MMP-9 Assay Kit (Cat. No E0321Ra). Lesion size and macrophage quantity were evaluated using histopathological analysis with hematoxylin and eosin (HE) staining. This study was conducted at the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada, Yogyakarta, Indonesia, from February to June 2023.

Plant material and extract preparation

The 60% ethanol extract of *P. niruri* was obtained from the Center for Research and Development of Medicinal Plants and Traditional Medicine (BPTOOT), Tawangmangu, Indonesia. The extraction process of *P. niruri* used the maceration method with 60% ethanol solvent. The *P. niruri* extraction process involved several steps. First, the *P. niruri* raw material was ground into a fine powder. Next, the powdered material was placed into an Erlenmeyer flask and mixed with a 60% ethanol solvent. The mixture was then vigorously shaken for 2–3 hours. Following this, the maceration process took place, during which the mixture was soaked and covered with aluminum foil for 24 hours. Finally, the mixture underwent filtration to separate the liquid from the solid components. The resulting filtrate was represented as a concentrated extract devoid of any solvent residue.

Animal care and treatment

A model of autologous endometriosis was developed using female Wistar rats sourced from the Laboratory of Integrated Research and Testing (LPPT) at Universitas Gadjah Mada, Yogyakarta, Indonesia. A total of 40 female Wistar rats (*Rattus norvegicus*) aged 6–12 weeks and weighing between 150 and 200 grams were used. The inclusion criteria required the animal to have a normal estrous cycle of 4–7 days and to be in healthy condition, characterized by the absence of fur loss, clear eyes, and active movement. The rats were acclimatized for 10 days, divided into five groups, and accommodated in standard cages with four rats per cage, considering their natural physiological and behavioral requirements. Rats with any signs of illness or infection during the adaptation period were excluded. The procedure for establishing the endometriosis model followed the protocol outlined by Amaral *et al.*, where endometriosis was induced by suturing a 0.5 cm^2 flap from the uterine horn to the peritoneal cavity [12]. The procedure began with anesthesia using pentobarbital sodium at a dose of 50 mg/kg BW administered intramuscularly. Subsequently, aseptic and antiseptic procedures are performed in the rat's abdomen to maintain cleanliness. A 4 cm midline incision was made below the center of the abdomen, and the bleeding was carefully controlled. The bicornuate uterus was identified, and a 0.5×0.5 cm section of the uterus was resected, involving both the endometrium and the serosal side. The rat's uterus was stitched to the right anterior peritoneum along a 3 cm line, with an incision facing the endometrial side towards the peritoneum. After ensuring there was no bleeding, the abdominal wall was closed with a single layer of PGA suture (No. 2). The reoperation was performed one month after the initial procedure to confirm the success of the endometriosis model, as indicated by the presence of an endometrial gland under histopathological observations.

Treatment procedure

The preparation of the therapeutic solution was carried out by dissolving *P. niruri* extract in Na-carboxymethyl cellulose and adding distilled water. The endometriosis rats were then given the solution for 14 days, with doses of 196 mg of *P. niruri* extract per 200 g BW for the PN 196 group, 392 mg of *P. niruri* extract per 200 g BW for the PN 392 group, and Dismeno was administered at a dose of 5.4 mg per 200 g BW for the Dismeno group. The solution was administered orally once daily as a single dose using a nasogastric tube. The allocation of rats to different groups was performed by a technician who was blinded to the specific treatments. The researchers conducting the treatments were also blinded to group allocations.

Rat dissection and tissue extraction

On day 46, all groups of rats were sacrificed by cervical dislocation and subsequently dissected. The rats were placed on a surgical board for abdominal dissection, and their spleens were harvested. Each tissue was fixed using formalin, then dehydrated with graded alcohol and cut into

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small pieces suitable for embedding in paraffin blocks for hematoxylin and eosin (H&E) staining. The researchers performing outcome assessments were blinded to group allocations.

Measurement of biomarkers

The enzyme-linked immunosorbent assay (ELISA) method was used to measure the levels of MDA, MMP-9, and IL-1β. All reagents, standards, and serum samples were left to reach room temperature. Strips were inserted into the frame, with unused strips stored at 2–8°C. The standard solution (50 μL) and the serum sample (40 μL) were added to their designated wells. Into the sample wells, 10 μL of the marker (IL-1β, MMP-9, or MDA) was added. Rat antibody and streptavidin-HRP (50 μL) were subsequently added to the sample and standard wells. The mixture was well-mixed, covered, and incubated for 60 minutes at 37°C. The cover was removed, and the plate was washed five times with 300 μL of wash buffer, soaking each well for 30 seconds to one minute. The plate was dried with absorbent material. Thereafter, substrate solution A (50 μL) was added, followed by substrate solution B (50 μL) into each well. The plate was then covered and incubated for 10 minutes at 37°C in the dark. Stop solution (50 μL) was added to each well, changing the color from blue to yellow. The optical density was measured at 450 nm using a microplate reader.

Number of macrophages

Preparations were made and stained with HE according to standard protocols to count the number of macrophages. The preparations were placed on microscope slides, and macrophage cells were identified under a microscope based on their characteristic morphology in the HE-stained preparations. The number of macrophages was counted using a microscope with a magnification of 400× in five fields of view. Desired images are captured by pressing caps and rec, using the ToupLite application for photo capture. The number of macrophages was calculated using the image roster application program. The count results were recorded and the average number of macrophages from the five fields of view was calculated for further analysis.

Determination of endometriosis lesion area

In the histological examination of the lesion area, the first step was to connect the computer to the microscope and run the image roster program. The preparation was placed under the microscope's objective lens, and desired images were captured using the ToupLite application and then saved in JPG format.

Statistical analysis

The normality of the data was tested using the Shapiro-Wilk test because the sample size was below 50. Data was considered normally distributed if $p>0.05$. If the data was normally distributed, analysis of IL-1β, MMP-9, MDA, number of macrophages, and lesion area was conducted using one-way ANOVA followed by a post hoc least significant difference (LSD) test. If the result of the normality test indicated a non-normal distribution, the Kruskal-Wallis test was employed, followed by the Mann-Whitney post hoc test to observe differences between groups. The significance level used was $\alpha=0.05$. The analyses were performed using the SPSS software version 27 for Windows (IBM, New York, USA).

Results

Effect of *Phyllanthus niruri* therapy on IL-1β levels

The changes in IL-1β levels before and after the treatment are presented in **Table 1**. Although the overall decrease in IL-1β levels post-treatment was not statistically significant across all groups ($p>0.05$), comparisons between groups revealed significantly lower IL-1β levels in the PN 196 group compared to the negative control group ($p=0.007$) and in the Dismeno group relative to the negative control group ($p=0.004$).

Effect of *Phyllanthus niruri* therapy on MDA levels and MMP-9 levels

The changes in MDA and MMP-9 levels before and after treatment are presented in **Table 1**. A significant decrease in MDA levels after the treatment was observed in the PN 392 group ($p=$

0.033). Additionally, we found significantly lower levels of MDA after the treatment in the PN 196 group (p=0.021) and the PN 392 group (p=0.012) compared to the negative control group. The level of serum MMP-9 significantly decreased in the PN 196 group (p=0.032). After the treatment, MMP-9 levels were significantly lower in the PN 196 group (p=0.021) and PN 392 group (p=0.012) compared to the negative control group.

Table 1. Effects of *Phyllanthus niruri* (PN) therapy on IL-1β, MDA, and MMP-9 levels

Biomarkers	Before PN therapy, mean±SD	After PN therapy, mean±SD	p-value [a] (before-after PN therapy)	p-value [c] (between groups)
IL-1β (Nmol/mL)				
Normal group	7.52±0.26	7.49±0.46	0.861	0.236 [d]
Negative control group	7.68±0.49	7.87±0.53	0.150 [b]	Ref.
PN 196 group	7.21±0.54	6.89±0.55	0.186	0.004*
PN 392 group	7.77±0.34	7.53±0.50	0.385	0.172 [d]
Dismeno group	7.33±1.27	6.96±0.99	0.153	0.007*
MDA (Nmol/mL)				
Normal group	1.01±0.07	1.03±0.08	0.742	0.021 [d]*
Negative control group	1.09±0.18	1.16±0.19 [c]	0.021 [b]	Ref.
PN 196 group	1.05±0.77	0.94±0.12	0.065	0.021 [d]*
PN 392 group	1.02±0.68	0.96±0.04	0.033	0.012 [d]*
Dismeno group	1.10±0.21	1.09±0.22 [c]	0.940	0.834 [d]
MMP-9 (Nmol/mL)				
Normal group	1.19±0.24	1.15±0.40	0.018	0.040 [d]*
Negative control group	1.15±0.17	1.24±0.15	0.012 [b]	Ref.
PN 196 group	1.14±0.06	1.08±0.11	0.032	0.021 [d]*
PN 392 group	1.15±0.05	1.11±0.03	0.102	0.012 [d]*
Dismeno group	1.16±0.25	1.09±0.17	0.404	0.074 [d]

[a] Analyzed using paired Student's t-test
[b] Analyzed using Wilcoxon test
[c] Analyzed using Mann-Whitney post hoc test
[d] Analyzed using post hoc least significant difference (LSD) test
* Statistically significant at p<0.05

Effect of *Phyllanthus niruri* therapy on macrophages and endometriotic lesions

Macrophage counts and endometriosis lesion area observed after the treatment are presented in **Figure 1**. Compared to the negative control group, significantly lower macrophage counts were observed in the PN 196 (p=0.003), PN 392 (p=0.005), and Dismeno (p=0.003) treatment groups. No significant difference in lesion area was observed between treated and untreated endometriotic rats. Histopathological images of the macrophage and endometriotic lesions are presented in **Figure 2**.

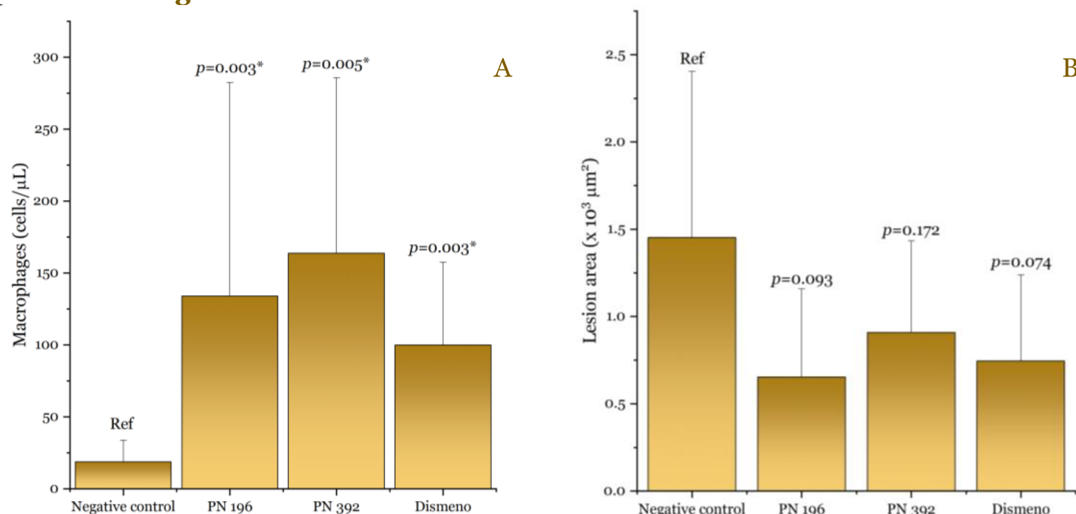

Figure 1. Macrophage counts (A) and lesion area (B) after the treatment. *Statistically significant at p<0.01 based on the Mann-Whitney test.

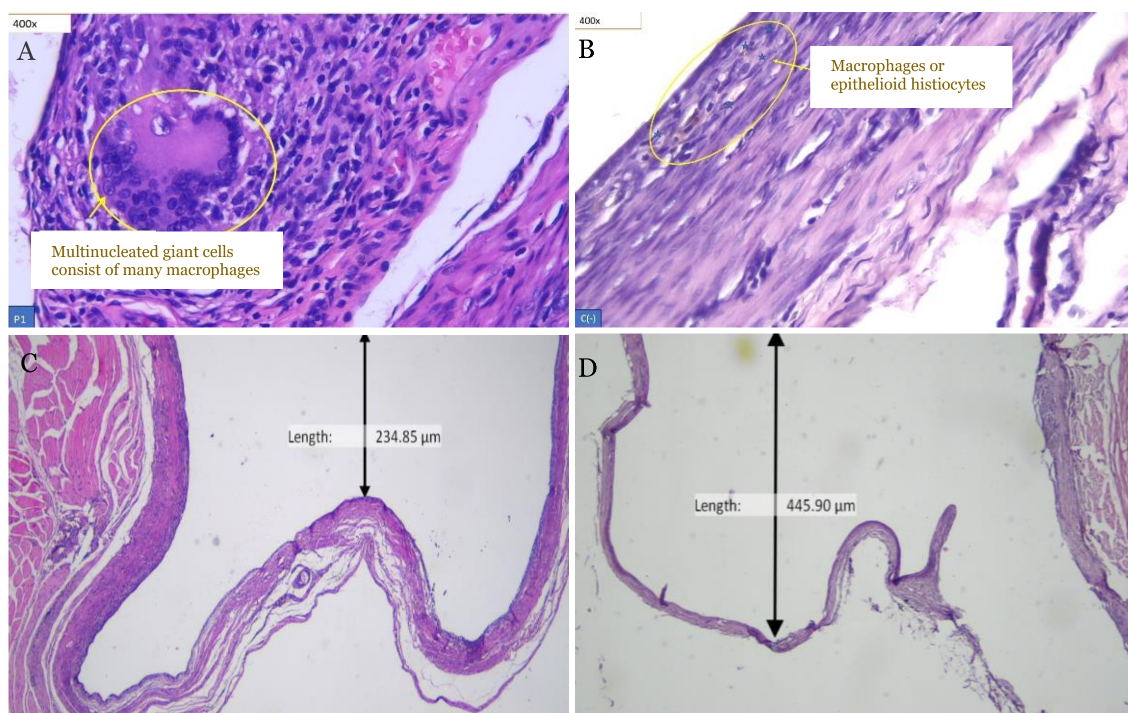

Figure 2. Photographed images of macrophages on hematoxylin and eosin (H&E)-stained slide in the PN 392 group (A) and the negative control group (B). Photographed images of the lesion area on a HE-stained slide in the PN 392 group (C) and the negative control group (D).

Discussion

The present study found that *P. niruri* therapy could attenuate IL-1β levels compared to negative control. A significant reduction of MDA was observed following the treatment. Post-treatment MDA levels among *P. niruri*-treated groups were significantly lower compared to the negative control group. Moreover, we found that the *P. niruri* therapy is significantly more effective in improving MDA levels than the commercialized *P. macrocarpa* extract. Levels of MMP-9 could be reversed by both the *P. niruri* and commercial *P. macrocarpa* extracts. Previously, the ability of tannins and flavonoids deriving from Phyllanthus species showed excellent affinity for IL-1β. Notably, niruriflavone and epigallocatechin had high docking scores of -50.23 and -50.16 kcal/mol, respectively, demonstrating their potential as alternative therapies for inhibiting IL-1β [13]. Herbecetin isolated from *P. niruri* has been reported to inhibit the release of proinflammatory cytokines, including TNF-α and IL-1β [11]. A previous study suggested that the strong antioxidant activity of *P. niruri* extract was attributed to the presence of quercetin, epicatechin, and rutin contents [14]. *P. niruri*-derived (-)-epicatechin has been reported to reduce the MDA level along with antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase [15]. In terms of its activity against MMP-9, it can be associated with the rich contents of gallic acid and quercetin, though other bioactive compounds might also take part in the activity [16]. The inhibition of MMP-9 by compounds from *Phyllanthus* spp. is reported to involve nuclear factor kappa B (NF-κB), mitogen-activated protein kinases, and phosphatidylinositol 3-kinase/Akt pathways [16].

IL-1β, MDA, and MMP-9 are considered as the promising therapeutical targets for endometriosis. The expression of IL-1β has been widely associated with endometriosis pathomechanism involving inflammatory reactions [17]. Moreover, IL-1β has a crucial role in the pathological processes of endometriosis. At the molecular level, IL-1β can trigger inflammatory responses by activating NF-κB as the primary regulator in the inflammatory response[18]. Previous studies reported a correlation between oxidative stress levels and the severity of endometriosis [19,20]. MDA is a biomarker for oxidative stress; thus, its decrease indicates the improvement of oxidative stress. As for MMP-9, the molecule has been suggested as the promising drug target, considering the crucial role of this proteolytic enzyme in the breakdown and remodeling of the extracellular matrix. Increased MMP-9 activity can lead to the degradation

of the extracellular matrix around endometriosis lesions, thereby facilitating the invasion and spread of endometriosis cells into surrounding tissues [21,22]. In line with a previous study, *P. niruri* extract was found to possess an anti-angiogenic effect on breast cancer in BALB/c mice [23]. Another study showed that *P. niruri* exhibits an antimetastatic effect, including the reduction of MMP-9 expression [24].

Findings from the present study also indicated that therapy with *P. niruri* resulted in higher macrophage counts as compared to normal and negative control groups. As for the endometriotic lesions, the effect of *P. niruri* extract appeared to be subclinical. This finding is intriguing because an increased macrophage count has often been associated with the severity of endometriosis in several previous studies. However, in our study, the increase in macrophage count was accompanied by improved biomarkers, suggesting that the therapeutic effects of *P. niruri* might be more complex and not solely dependent on macrophage count, but also on the phenotype and function of these macrophages [25,26,27]. Previous studies have demonstrated that *P. niruri* extract enhanced the activity of the cytokine IFN-γ released by Th1 cells, leading to the activation of M1 macrophages that indicates an inflammatory condition [28]. Additionally, other studies have shown that the combination of *P. niruri* and *C. roseus* extracts suppressed macrophage polarization to the M2-type, which possesses anti-inflammatory properties, while simultaneously promoting macrophage polarization to the M1-type, which has pro-inflammatory activity [10]These findings suggested that the therapeutic effect of P. niruri in endometriosis is complex and may involve modulating macrophage function rather than simply reducing macrophage count. Given the results obtained from macrophage examination using H&E staining, the examination aspect needs to be considered. Better results may be obtained using an immunohistochemistry examination.

The limitations of this study should be considered when interpreting the findings. Firstly, this study only utilized *P. niruri* crude extract without compound isolation. A more targeted approach could yield more favorable outcomes, such as using isolated active compounds or specific extract fractions. Secondly, the study primarily relied on biomarker and histopathological analysis, which may not fully capture the complex mechanisms involved in endometriosis and the therapeutic effect of *P. niruri*. Future studies could incorporate molecular analyses, such as gene expression profiling or proteomic analysis, to elucidate the underlying pathways affected by *P. niruri* therapy. Additionally, this study cannot be generalized to humans since it used a rat model. Lastly, while the results are promising, long-term safety and potential side effects of prolonged *P. niruri* administration should be thoroughly investigated in future studies.

Conclusion

P. niruri extract could attenuate the inflammatory responses in the endometriosis rat model, as evidenced by decreased levels of IL-1β and MMP-9. Moreover, the extract could reduce oxidative stress, as indicated by decreased levels of MDA. Additionally, the extract was evident to decrease the extent of endometriosis lesions, although it did not reduce macrophage quantity. For future studies, we recommend conducting a more thorough observation using immunohistochemical analysis and evaluating the phagocytic activity of the macrophages.

Ethics approval

The research ethical protocol was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia (KE/FK/0093/EC/2023).

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

How to cite

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