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Effects of andrographolide on p53 and Ki67 expression in an experimental mouse model of endometriosis

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Academic editor

Hasan-Al-Faruque, PhD
University of Utah
USA

Article info

Received: 27 May 2026

Accepted: 30 July 2026

Published: 18 August 2026

Keywords

Andrographolide, Endometriosis,
Mouse model, Ki67, P53

ABSTRACT

Endometriosis is a long-term condition that causes inflammation and leads to the growth of abnormal tissue outside the uterus. This tissue can cause significant problems, including pain and infertility. Researchers have been seeking new ways to treat endometriosis, and one promising treatment option is andrographolide. This compound comes from a plant called *Andrographis paniculata* and has been shown to stop the growth of abnormal cells and promote cell death. To learn more about how andrographolide works, the current study conducted an experiment using mice with endometriosis. The mice were divided into six groups: a normal group, an endometriosis group that didn't receive any treatment, a group that received a standard treatment, and three groups that received different doses of andrographolide. Tissue samples from each group were examined to see how the treatment affected the growth of abnormal cells. The mice that received andrographolide had lower levels of certain proteins, such as p53 and Ki67, which are associated with cell growth and division, respectively. Andrographolide might be able to slow down the growth of abnormal tissue in endometriosis. However, the differences between the andrographolide groups and the standard treatment group were not statistically significant. Despite this, the study suggests that andrographolide might be a useful treatment for endometriosis, particularly for women who prefer not to use hormonal therapies. More research is needed to determine the optimal dose and treatment schedule, but the results are promising. The fact that andrographolide appears to target specific molecular markers associated with endometriosis makes it a potentially powerful tool for treating this condition. With further development, andrographolide could become a valuable treatment option available to women with endometriosis.

INTRODUCTION

Endometriosis is a condition in which tissue similar to the lining of the uterus grows outside the uterus, often on the pelvic peritoneum. This can cause chronic pain and infertility. The exact causes of this condition are not fully understood, but it's thought to be related to an imbalance between the growth and death of cells in the affected tissue. Hormone treatments such as dienogest are often used, but they can have long-term side effects, and the condition can persist. Thus, there is an unmet need for exploring other treatments that target specific molecules [1, 2].

Andrographolide is a distinctive compound identified in the plant *Andrographis paniculata*. It has been shown to possess anti-inflammatory properties, thereby assisting in the reduction of swelling and pain. Furthermore, it exhibits antiproliferative properties, indicating its potential to inhibit excessive cellular growth. Additionally, it demonstrates pro-apoptotic properties, facilitating programmed cell death when cells are no longer required. Research has indicated that andrographolide can decelerate cell



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proliferation by diminishing the production of specific proteins, such as Ki67. Ki67 is a protein frequently utilized as a marker of cellular proliferation, thereby providing insights into the rate of cellular growth [3-5]. Andrographolide has also been shown to stop the growth of abnormal cells by affecting certain pathways in the cell, such as the NF- κ B and MAPK pathways. These pathways are important for cell growth and inflammation. Endometriosis is a condition where tissue similar to the lining of the uterus grows outside of the uterus, often causing pain and discomfort. Cell proliferation is a key feature of endometriosis, and Ki67 is often used as a marker of this proliferation [6, 7]. Several studies have found that Ki67 expression is higher in endometriotic tissue than in normal tissue. This suggests that cells in endometriotic tissue grow more quickly than normal cells [8]. These findings are supported by Xiao *et al.*, who reported a significant increase in Ki67 expression in peritoneal endometriosis lesions. Consistently, several studies observed that elevated cell proliferation indices contribute to the progression of endometriosis lesions [9-11].

In the context of endometriosis, numerous preclinical studies have demonstrated that andrographolide can effectively reduce both the size and number of endometriotic lesions in animal models. This effect is believed to be linked to a decrease in Ki67 expression, indicative of reduced cell proliferation, alongside an increase in p53 expression, which reflects the activation of apoptosis. However, not much is known about how andrographolide affects Ki67 and p53 expression in the peritoneal tissue of mice with endometriosis. Furthermore, the anti-inflammatory properties of andrographolide contribute to a reduction of the peritoneal inflammatory environment that facilitates the growth of endometriosis lesions [12, 13]. This study represents the first experimental investigation to directly compare the effects of andrographolide with standard dienogest therapy on Ki67 and p53 expression in a Balb/C mouse peritoneal endometriosis model.

Considering all of the above, the current study aimed to evaluate the potential of andrographolide as an alternative therapy for endometriosis by modulating the balance between proliferation and apoptosis, as indicated by Ki67 and p53 expression in peritoneal tissue.

MATERIALS AND METHODS

Experimental animals

This experimental investigation utilized a post-test-only control group design involving 42 healthy female Balb/C mice, aged 8–10 weeks and weighing 20–30 g, obtained from the Animal Engineering Laboratory, Faculty of Veterinary Medicine, University of Airlangga, Surabaya, Indonesia. The animals were acclimated for 1 week before the experiment and maintained under controlled environmental conditions ($22 \pm 2^\circ\text{C}$, 50–60% relative humidity, 12-hour light/dark cycle), with unrestricted access to standard laboratory chow diet and water *ad libitum*.

All experimental procedures were approved by the Medical Research Ethics Committee of the Faculty of Medicine, Diponegoro University (Approval No. 108/EC/KEPK/FK-UNDIP/XI/2024).

Experimental endometriosis mouse model and treatment protocol

To establish the experimental endometriosis model, mice received intramuscular injections of ethinyl estradiol (0.2 μg) to induce lesions, followed by intraperitoneal

implantation of syngeneic endometrial tissue. Cyclosporine A (0.2 mL) was administered to suppress immune rejection and facilitate lesion development. Fourteen days after implantation, successful establishment of endometriotic lesions was confirmed macroscopically.

Animals were randomly assigned into six groups ($n = 7$ per group), including N (no treatment), KN (endometrial cells), KP (endometrial cells + dienogest 0.005 mg/kg), P1 (endometrial cells + andrographolide 0.05 mg/kg), P2 (endometrial cells + andrographolide 0.1 mg/kg), and P3 (endometrial cells + andrographolide 0.2 mg/kg). Treatments were administered according to the assigned groups throughout the experimental period for 14 days.

On the day of 15th, before euthanizing the mice with non-inhalation agents, preparations for peritoneal lavage macrophages for flow cytometry began. These macrophages were first isolated from the peritoneal cavity and carefully processed to remove debris and non-cellular materials. The peritoneal lavage was collected, and fluorochromes were used for cell isolation, followed by analysis with phosphate-buffered saline (PBS). Two centrifugation steps at 2500 rpm were then conducted. This centrifugation method separates components within the lavage fluid based on density, eliminating the need for nylon mesh filtration because of the high purity of the macrophage suspension. Different centrifugation speeds and durations can be used to pellet the cells, debris, or other particles, allowing collection of either supernatants or pellets.

Sample collection and laboratory assay

Before the mice were euthanized, peritoneal fluid was collected in PBS and then centrifuged twice at 2500 rpm. The abdominal wall and peritoneum were separated; the peritoneum was then cut, stretched on graph paper, and photographed to assess the extent of endometriosis. The results were recorded on a data collection sheet and statistically analyzed.

Preparations for anatomical pathology examination were performed using the reddest peritoneal tissue, which was collected as specimens and fixed in 10% formalin. The peritoneum was examined using a Nikon H600L microscope equipped with a 300-megapixel DS-Fi2 digital camera and Nikon image-processing software (Nikon Corporation, Tokyo, Japan). The endometriosis implantation sites were assessed macroscopically in areas of hyperemia, and samples were collected from the most hyperemic areas to confirm the presence of endometriotic lesions. This study compared the formation of ectopic endometrial lesions in Balb/C mice treated with andrographolide at various therapeutic doses to those in a normal group, endometriosis group, and a positive control group receiving standard drug therapy. Peritoneal fluid collection was performed 14 days after intraperitoneal injection of syngeneic endometrial tissue.

Tissue collection and histopathological evaluation

After collection of peritoneal fluid, macroscopic endometriotic lesions were identified based on hyperemic appearance, photographed, and measured. Tissue samples from the most hyperemic lesions were fixed in 10% neutral buffered formalin, processed routinely, embedded in paraffin, sectioned, and subjected to immunohistochemical examination.

Immunohistochemical staining for Ki67 and p53 was performed using standard laboratory procedures. Stained slides were independently evaluated by two blinded anatomical pathologists using the modified Remmele Immunoreactive Score (IRS), which combines the percentage of positively stained cells with staining intensity to generate a semi-quantitative score. Inter-observer agreement was assessed using Cohen's kappa coefficient (κ) to determine scoring reliability. Immunohistochemical expression of Ki67 and p53 was evaluated semi-quantitatively using the modified Remmele IRS system. The IRS score was calculated by multiplying the percentage score of positively stained cells by the staining intensity score, resulting in a composite score reflecting the degree of biomarker expression.

To assess inter-observer reliability between the two blinded anatomical pathologists, inter-rater reliability (IRR) analysis was performed using κ . Kappa values were interpreted according to established criteria as follows: $\kappa < 0.20$, indicating slight agreement; 0.20–0.40, fair agreement; 0.41–0.60, moderate agreement; 0.61–0.80, substantial agreement; and 0.81–1.00, almost perfect agreement. Higher kappa values indicated stronger consistency and reliability between observers in assessing immunohistochemical staining results.

Statistical analysis

Statistical analyses were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Data were expressed as mean $\pm$ standard deviation for normally distributed variables or median (minimum–maximum) for non-normally distributed variables. Normality was assessed using the Shapiro–Wilk test. Comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by an appropriate post hoc multiple comparison test for normally distributed data, whereas the Kruskal–Wallis test was used for non-parametric variables. Comparisons between two independent groups were analyzed using the independent-samples t-test or the Mann–Whitney U test, as appropriate. Statistical significance was defined as a two-sided p-value < 0.05 .

RESULTS

Effect of andrographolide on inter-rater reliability of immunohistochemical scoring in the endometriosis mouse model

Immunohistochemical analysis of p53 and Ki67 was successfully performed on all samples. The quality and consistency of immunohistochemical scoring were evaluated through inter-rater reliability analysis between two blinded pathologists. Cohen's kappa coefficients demonstrated excellent agreement across all biomarkers, confirming the reliability and reproducibility of the scoring method. Specifically, p53 exhibited kappa values ranging from 0.960 to 1.000, and Ki67 from 0.905 to 0.997 (Table 1). These findings validate the robustness of the semi-quantitative IRS assessments used throughout the study.

Table 1. Cohen's Kappa Coefficient for p53 and Ki67 between the two anatomical pathologists.

	Cohen's Kappa Coefficient	Sig.
p53		
Group N	0.973	0.000
Group KN	0.997	0.000
Group KP	1	-
Group P1	0.996	0.000
Group P2	0.978	0.000
Group P3	0.960	0.000
Ki-67		
Group N	0.905	0.000
Group KN	0.940	0.000
Group KP	0.997	0.000
Group P1	0.931	0.000
Group P2	0.992	0.000
Group P3	0.997	0.000

N of Valid Cases 42
N (no treatment), KN (endometrial cells), KP (endometrial cells + dienogest 0.005 mg/kg), P1 (endometrial cells + andrographolide 0.05 mg/kg), P2 (endometrial cells + andrographolide 0.1 mg/kg), and P3 (endometrial cells + andrographolide 0.2 mg/kg). n=42.

Effect of andrographolide on p53 expression in the endometriosis mouse model

Evaluation of p53 expression using the IRS method showed higher expression in the untreated endometriosis group than in the normal group. The endometriosis group had the highest p53 IRS value (4.11 ± 1.06), whereas the normal group had lower baseline expression (2.51 ± 1.04). Treatment with andrographolide gradually reduced p53 expression in a dose-dependent manner. Notably, the highest-dose andrographolide group (P3) had the lowest p53 expression (Table 2).

Microscopic examination supported the quantitative findings. Figure 1 shows representative immunohistochemical staining of p53 in peritoneal endometriotic lesions. The brown chromogen indicates positive p53 expression, mainly in the nuclei of endometriotic stromal and glandular cells at 400× magnification. The untreated endometriosis group exhibited more widespread and intense nuclear staining compared to the normal group, suggesting possible dysregulation of apoptosis pathways in ectopic endometrial tissue. In contrast, the groups treated with andrographolide, especially P2 and P3, displayed noticeably weaker staining and fewer positively stained nuclei. This indicates that these treatments may influence apoptosis-related mechanisms and suppress endometriotic cell activity.

Table 2. The IRS expression of p53.

Group	IRS expression of p53		
	Mean±SD	Median	(Min - Max)
N	2.51±1.04	2.00	(1.60-4.35)
KN	4.11±1.06	4.50	(2.30-5.50)
KP	3.60±1.84	3.30	(1.50-7.20)
P1	3.18±1.14	2.90	(2.00-4.90)
P2	2.74±1.06	2.90	(1.20-4.10)
P3	2.51±0.89	2.30	(1.20-4.00)

N (no treatment), KN (endometrial cells), KP (endometrial cells + dienogest 0.005 mg/kg), P1 (endometrial cells + andrographolide 0.05 mg/kg), P2 (endometrial cells + andrographolide 0.1 mg/kg), and P3 (endometrial cells + andrographolide 0.2 mg/kg). n=42.

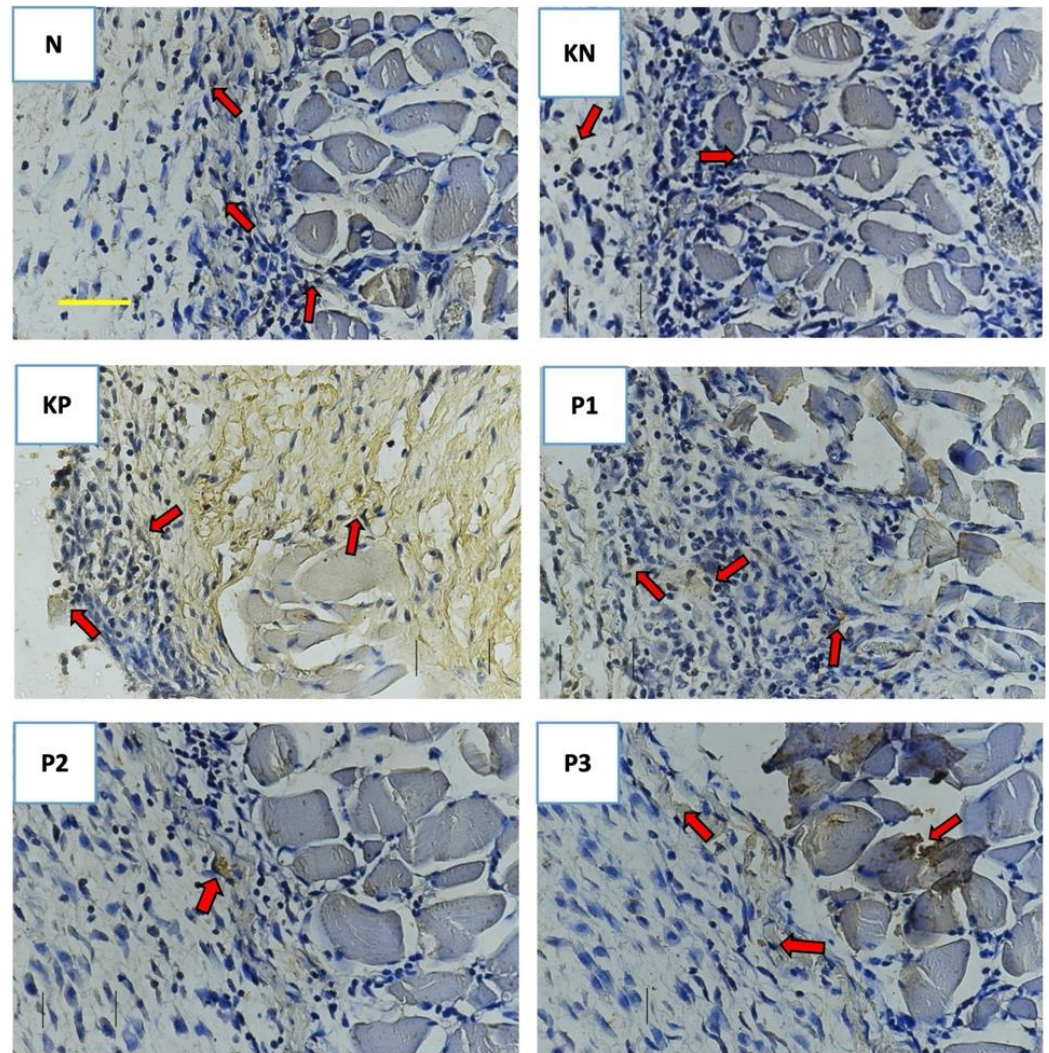


Figure 1. The arrow indicates the presence of p53 expression in endometriosis tissue (peritoneum), which is indicated by the presence of brown chromogen color (red arrow) in IHC. 400 (n=42). Scale bar = 50 μ m. N (no treatment), KN (endometrial cells), KP (endometrial cells + dienogest 0.005 mg/kg), P1 (endometrial cells + andrographolide 0.05 mg/kg), P2 (endometrial cells + andrographolide 0.1 mg/kg), and P3 (endometrial cells + andrographolide 0.2 mg/kg).

Effect of andrographolide on Ki67 expression in the endometriosis mouse model

Ki67 analysis showed similar trends. The endometriosis group had higher Ki67 levels than the normal group, indicating increased proliferation in ectopic tissue. Mean IRS scores were 2.90 ± 1.73 for the endometriosis group and 2.20 ± 1.49 for the normal group. The dienogest group had elevated Ki67 (3.60 ± 1.84). Treatment with andrographolide dose-dependently reduced Ki67, especially at higher doses (Table 3).

Histopathological observations supported the semi-quantitative IRS results (Figure 2). Ki67-positive cells showed brown nuclear staining in actively proliferating endometriotic cells. The untreated endometriosis group displayed widespread, intense staining compared to the normal group, indicating higher proliferative activity linked to lesion progression. Conversely, groups treated with andrographolide showed gradual decreases in both staining intensity and the number of Ki67-positive nuclei with increasing doses. The most significant declines were seen in the P2 and P3 groups, indicating effective inhibition of cellular proliferation in ectopic endometrial lesions.

Table 3. The IRS expression of Ki67.

Group	IRS expression of Ki67		
	Mean±SD	Median	(Min - Max)
N	2.20±1.49	2.10	(0.30-3.90)
KN	2.90±1.73	2.90	(0.30-5.20)
KP	3.60±1.84	3.30	(1.50-7.20)
P1	3.52±1.61	3.70	(0.80-6.00)
P2	2.57±0.81	2.50	(1.80-3.90)
P3	2.45±1.35	2.30	(0.80-4.30)

N (no treatment), KN (endometrial cells), KP (endometrial cells + dienogest 0.005 mg/kg), P1 (endometrial cells + andrographolide 0.05 mg/kg), P2 (endometrial cells + andrographolide 0.1 mg/kg), and P3 (endometrial cells + andrographolide 0.2 mg/kg). n=42.

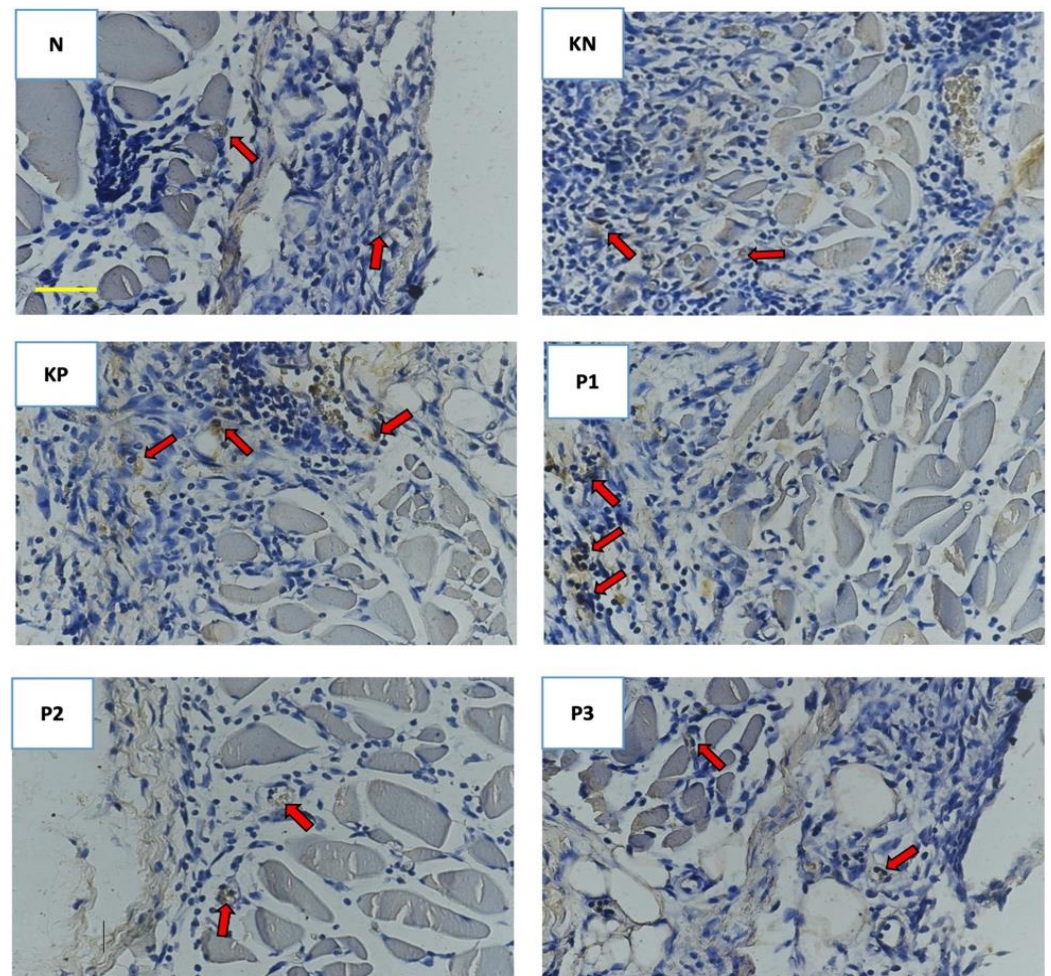


Figure 2. The arrow indicates the presence of Ki67 expression in endometriosis tissue (peritoneum) as indicated by the presence of brown chromogen (red arrow). IHC. 400x (n=42). Scale bar = 50 µm. N (no treatment), KN (endometrial cells), KP (endometrial cells + dienogest 0.005 mg/kg), P1 (endometrial cells + andrographolide 0.05 mg/kg), P2 (endometrial cells + andrographolide 0.1 mg/kg), and P3 (endometrial cells + andrographolide 0.2 mg/kg).

Effects of andrographolide on proliferation–apoptosis regulation in the endometriosis mouse model

Both p53 and Ki67 analyses showed a dose-dependent reduction in biomarker levels after andrographolide treatment. While differences weren't always statistically significant, the combined findings suggest that andrographolide affects pathways involved in cell growth and death in endometriosis. This indicates it could suppress lesion activity and serve as a non-hormonal, targeted therapy.

DISCUSSION

In examining the development of endometriosis, it is important to recognize that it involves not merely excessive cell proliferation. The process of programmed cell death, known as apoptosis, also becomes disrupted. A protein called p53 functions as a safeguard of the cell cycle and facilitates cell death when the DNA sustains damage [14-16]. However, in cases of endometriosis, the p53 protein does not function properly. Several studies have demonstrated that the expression of p53 protein is markedly reduced in endometriotic tissue, implying that cellular apoptosis is impaired. For instance, a study conducted by Duan *et al.* revealed that the majority of endometriosis lesions exhibited very low or undetectable levels of p53 protein [17]. This indicates that the cells within these lesions lack normal apoptotic capacity, thereby potentially contributing to the progression of endometriosis [17]. Andrographolide is a compound derived from the plant *Andrographis paniculata*. It is recognized for its anti-inflammatory properties, its ability to inhibit excessive cellular proliferation, and its role in promoting apoptosis when appropriate. Scientific investigations have demonstrated that andrographolide can impede cellular growth by decreasing the expression of specific markers, such as Ki67. Additionally, it can inhibit the proliferation of abnormal cells by modulating the cell cycle and key signaling pathways, including NF- κ B and MAPK, which are integral to cellular proliferation and inflammatory responses [18-20].

This study revealed that andrographolide is able to decrease the activity of specific proteins like p53 and Ki67 in a mouse model of endometriosis. The reduction in activity was proportional to the dosage of andrographolide. Microscopic examination showed decreased staining and fewer actively dividing cells, especially at higher doses. This indicates that andrographolide may influence the growth of endometriosis lesions and the regulation of cell death crucial, given that endometriosis involves excessive cell proliferation, insufficient apoptosis, inflammation, and tissue alterations. These results suggest andrographolide's potential as a non-hormonal treatment for endometriosis. As a proliferation marker, Ki67 is often elevated in ectopic endometrial tissue, and untreated lesions exhibited significantly higher Ki67 levels, indicating increased growth [21-24].

Ki67 is a well-established marker of cellular proliferation and is frequently overexpressed in ectopic endometrial tissue, reflecting the enhanced proliferative activity characteristic of endometriosis. In the present study, andrographolide treatment was associated with a dose-dependent trend toward reduced Ki67 expression, suggesting a potential antiproliferative effect. Likewise, p53 expression showed a gradual reduction following andrographolide administration, although the differences were not statistically significant compared with the dienogest-treated group. These findings indicate that andrographolide may influence biological processes related to cell proliferation and apoptosis in endometriotic lesions. Previous experimental studies have reported that the antiproliferative and anti-inflammatory effects of andrographolide may involve modulation of signaling pathways such as NF- κ B, MAPK, and PI3K/Akt, as well as suppression of pro-inflammatory cytokine production. However, these molecular pathways were not directly investigated in the present study. Therefore, the involvement of these signaling mechanisms should be considered a plausible hypothesis based on published evidence rather than a conclusion supported by the current experimental data. Future studies incorporating molecular analyses, including assessment of NF- κ B, MAPK, PI3K/Akt signaling components, inflammatory cytokines, and angiogenic markers, are needed to clarify the mechanisms by which andrographolide modulates proliferation and apoptosis in endometriosis.

The expression of p53 presented a more complex picture; it was highest in untreated lesions and declined after treatment, which may suggest reduced stress and inflammation rather than a direct impact on apoptosis. Additional markers will be necessary for a more comprehensive understanding. The dose-dependent reduction observed supports the activity of andrographolide. Additionally, it suppresses cytokines, inhibits angiogenesis, reduces oxidative stress, and modulates immune responses, all of which are relevant to endometriosis. These effects suggest a multi-pathway mechanism, providing a promising non-hormonal alternative to current hormone-based treatments that may have adverse effects.

The limitations are that the relatively small sample size may have diminished the statistical power to detect significant differences among the treatment groups; the duration of treatment was relatively brief and may not adequately reflect the long-term biological effects of andrographolide. Consequently, the molecular mechanisms behind the observed changes remain uncertain. Lastly, the semi-quantitative IRS method may introduce observer variability, even with excellent inter-rater agreement. Further research with larger samples and longer treatment.

CONCLUSIONS

In this experimental mouse model of endometriosis, andrographolide exhibited a dose-dependent trend toward diminishing Ki67 and p53 expression within peritoneal lesions. Nevertheless, these decreases did not reach statistical significance when compared to the standard treatment, dienogest. Consequently, the current findings imply that andrographolide may influence proliferation-and apoptosis-related processes in endometriotic tissue; however, the evidence remains insufficient to confirm its therapeutic efficacy. These preliminary results advocate for further research employing larger sample sizes, extended treatment durations, and supplementary molecular analyses to elucidate the biological mechanisms and potential therapeutic role of andrographolide in the context of endometriosis.

ACKNOWLEDGEMENTS

The authors acknowledge Prof. Dr. Widjiati, M.Si., Drh., drh. Suryo Kuncorojakti, and drh. Riski Lesta Mega for their esteemed support and contributions. We also acknowledge the Doctoral Program in Medical and Health Sciences, Universitas Diponegoro, Semarang; the Department of Embryology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya; the Institute of Tropical Disease, Universitas Airlangga, Surabaya; the Dexa Laboratory of Biomolecular Science, Cikarang; and the Faculty of Medicine, Soegijapranata Catholic University, Semarang, Indonesia.

AUTHOR CONTRIBUTIONS

IAS: conceptualization, data collection, and writing. IM: data analysis and writing. AKM: data collection, data analysis. AWK: conceptualization, writing. EH: conceptualization, writing. FPH: data analysis. All authors have read and approved the final manuscript.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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