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Original Research Article

Phaleria Macrocarpa Extract Reduced Endometriosis Lesions by Regulating Inflammatory Protein and Inhibiting Ki67 and Vegf-A Protein

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ABSTRACT

Endometriosis is a complex and multifactorial disease causing severe pelvic pain, infertility, and reduced quality of life in women. Therefore, this study aimed to evaluate the preventive potential of *Phaleria macrocarpa* (PM) mesocarp ethanol extract on endometriosis. The animals used for the experiment were divided into eight groups, each comprising four mice. The first group served as control comprising healthy mice without PM treatment and adenomyosis (ADM) implantation. The second group was the control negative consisting of mice with ADM implantation and without PM treatment. The remaining six groups consisted of ADM mice treated with several doses of PM including 3.75, 7.5, 11.25, 15, 18.75, and 22.5 mg/kg/day orally for 14 days. On day 1 PM treatment, endometriosis was induced by implanting ADM tissue intraperitoneally in mice given an immunosuppressant, cyclosporin. Furthermore, mice were injected with estradiol intramuscularly (54 IU/mice) on the first and fifth day after ADM implantation. At day 15, the samples were sacrificed, endometriosis tissue was isolated to measure the implant area, and the ascites of peritoneal were collected to evaluate the level of Cyclooxygenase-2 (COX-2). The evaluation was performed using Enzyme-Linked Immunosorbent Assay (ELISA), and Nuclear Factor kappa-light-chain-enhancer of activated B cells p65 subunit (NF-κB p65), Vascular Endothelial Growth Factor A (VEGF-A), and Kiel-67 (Ki67) of peritoneal tissue expression level by immunohistochemistry. The results showed that PM could prevent inflammation and the development of endometriosis by reducing the level of COX-2 in a dose-dependent method. Additionally, PM extract inhibited the expression level of NF-κB p65, VEGF-A, and Ki67, reducing the ADM implant area.

Keywords: Adenomyosis, Endometriosis, inflammation, *Phaleria macrocarpa*

Introduction

Endometriosis is a chronic and complex disease characterized by the appearance of endometrial-like tissue outside the uterus. This condition affects approximately 10-15 % of women of reproductive age worldwide[1]. Based on the lesion size, location, and tissue growth, endometriosis is classified into four stages, namely minimal, mild, moderate, and severe (I-IV stage, respectively)[2]. Endometriosis commonly causes two major clinical symptoms, including severe pelvic pain and infertility[3]. Associate symptoms mostly felt by endometriosis patients include cyclical and non-cyclical pelvic pain, dysmenorrhea, dyspareunia, dyschezia, and dysuria[4].

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However, it also reduces the patient's quality of life, manifested as both physical and mental[5]. As an inflammatory disease, endometriosis is caused by the dysfunction of the immune system. The alteration of the peripheral and endometrial immune system in women is associated with infertility, failure of early pregnancy, and atypical tissue homeostasis[6]. In the pathophysiology of endometriosis, immunocompetent cells such as neutrophils and monocytes are increased[7]. Furthermore, macrophages are essential in the pathogenesis of endometriosis, overproducing inflammatory cytokines and promoting lesion progression, proliferation, and angiogenesis[8]. Endometriotic cells, in response to interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and toll-like receptor 4 (TLR4), can activate the Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway and lead to the initiation and progression of endometriosis[9]. The initiation and progression of endometriosis through the NF-κB signaling pathway is related to vital regulatory factors such as estrogen, progesterone, oxidative stress, and noncoding RNAs (ncRNAs)[10]. The complexity of this disease and cause still need to be fully understood, as no curative treatment exists[1]. The treatment of endometriosis is palliative in easing pain and increasing quality of life[11]. In line with recent advancements, the available options for endometriosis treatment include OCPs (oral contraceptive pills), progestins, GnRH (gonadotropin hormone-releasing hormone) agonists, aromatase inhibitors, and GnRH antagonists[12]. However, the consumption of OCPs and GnRH agonists leads to the decline of anti-müllerian hormone (AMH) levels as the marker of ovarian reserve and

infertility[13]. This shows the need for a safer alternative treatment from medicinal plants such as *Phaleria macrocarpa* Boerl. (PM).

Phaleria macrocarpa Boerl. (PM) is an Indonesian medicinal plant also called mahkota dewa, with pharmacological properties for various disease treatments such as asthma, dysentery, and rheumatoid arthritis [14]. It is a species in the family Thymelaeaceae, geographically distributed around Papua and widely grown on Java Island, Indonesia. Previous studies have reported the bioactive compounds contained in the ethanolic extract of PM mesocarp, which includes eriodyctol, glycitin, 5-O-methyl genistein, (þ)-catechin 7-O-beta-D-xyloside, (-)-8-Prenylnaringenin, and (±)-Naringenin[15]. This extract can control endometriosis through several mechanisms, such as reduced expression of matrix metalloproteinase (MMP)-1[16,] MMP-3, MMP-7 [17], MMP-2, MMP-9, and NF-κB[18]. PM extract can also suppress endometriosis lesion growth by modulating proliferation and apoptosis[15], although its effect on endometrial inflammation remains unclear. Therefore, this study aimed to analyze the effect of PM extract on endometrial inflammation.

Materials and Methods

Plant Collection and Identification

The fruits of *Phaleria macrocarpa* were collected on November 2023 and the mesocarp was processed into powder in UPT Materia Medica Batu Malang, Indonesia (7°52'01.2"S and 112°31'13.2"E). The taxonomic identification of the plant was done and deposited by Prof. Dr.Syaukani, M.Sc, the head of Biosystem at UPT Materia Medica Batu with voucher number 49/UN11.1.8.4/TA.00.03/2023.

Extraction and Fractionation of PM Mesocarp

Extraction was conducted through the maceration method by soaking 2500 g of PM mesocarp powder in 96% ethanol (m;v, 1;12), with continuous stirring for 30 min, and waiting for five days. The filtration was done using a Buchner funnel to obtain the macerate. Subsequently, extract was evaporated using a rotary evaporator for 8 h at 60°C.

The fractionation was performed by weighing 2 g of ethanol extract of PM fruit mesocarp and dissolving in 30 ml of distilled water. Subsequently, the mixture was poured into the Buchner funnel, added, and homogenized with 70 ml n-hexane in the separating funnel. The water phase was isolated from the n-hexane phase which was added and homogenized with 70 ml n-butanol. The fractionation of each solvent was performed in triplicate and n-butanol phase was evaporated using a rotary evaporator for 8 h at 60°C to obtain concentrated extract for further analysis.

Animals

BALB/c mice (6-8 weeks old; 20-30 g in weight) with healthy and normal conditions were used in this study. The animals were maintained with access to food and water under a controlled environment (25°C temperature and 12 h cycle of light and dark) and acclimated for 1 week. Then, the animals were randomly divided into eight groups, each comprising four mice. The first group served as a control, which consisted of healthy mice without PM treatment and adenomyosis (ADM) implantation. The second group was ADM mice with ADM implantation and without PM treatment. Meanwhile, the remaining six groups were mice with ADM implantation and treatment with several doses of PM such as 3.75, 7.5, 11.25, 15, 18.75, and 22.5 mg/kg/day. This study was ethically approved by the Universitas Brawijaya Faculty of Medicine ethical committee (No. 67/EC/KEPK/03/2023).

Endometriosis induction

On day 1 of PM treatment, endometriosis mice model was given an immunosuppressant cyclosporin injection intramuscularly (0.2 ml/mice). After that, the immunodeficiency mice were implanted intraperitoneally with 0.1 ml of ADM tissue derived from patients. The tissue in size of 1 cm^3 was washed and suspended in 10 ml PBS and centrifuged at 2500 rpm and 4°C for 10 min[19]. On the first and fifth day after implantation, mice were intramuscularly injected with ethinyl estradiol (54 IU/mice). Subsequently, PM treatment was performed for 14 days, and on the next day, the mice were sacrificed through a cervical dislocation for further analysis.

Isolation and measurement of endometriosis lesion

Mice were sacrificed through cervical dislocation and the area of endometriosis lesion was assessed macroscopically in the hyperemic region. When endometriosis lesions were found, the area of implantation was measured by calculating the reddish lesion on the peritoneal wall, measured in square millimeters (mm^2) using the Motic Image software. The measurements are performed by three different individuals and averaged. This examination was intended to determine the area of the implant and hypervascularization in the peritoneal tissue of endometriosis mice model. The data for each sample was assessed macroscopically and measured using an image roster based on the presence of nodules/lesions and hypervascularization of blood vessels in the peritoneal tissue[20].

Enzyme-Linked Immunosorbent Assay (ELISA) analysis

The measurement of COX-2 level was analyzed using mice COX-2 ELISA Kit, by preparing the sample from the supernatant of centrifuged (2,000 rpm for 10 min) mice ascites. Subsequently, a 50 µl Human COX-2 standard or sample was added to the appropriate wells, followed by a 50 µl antibody cocktail. The washing buffer and 3,3′,5,5′-Tetramethylbenzidine (TMB) solution were added respectively before the stop solution (1N Sulfuric Acid (H_2SO_4), and the absorbance was read with optical density (OD) at 450 nm[21].

Immunohistochemistry analysis

The peritoneal tissue of mice was isolated and the expression of NF-κB p65, VEGF-A, and Ki-67 were evaluated through immunohistochemistry analysis. Initially, the lesion found in the peritoneal tissue was sliced for approximately 0.5 cm thickness and soaked for 24 h in 10% formalin. This was followed by using a serial dilution of alcohol to dehydrate the tissue and clear with xylol. The tissue was embedded in a paraffin block, sliced into 6 µm, and deparaffinized. The sample was stained with NF-κB p65, VEGF-A, and Ki-67 antibodies, and the image was captured using an AccuView microscope. The NF-κB p65, VEGF-A, and Ki-67 expressions were analyzed using ImageJ software[22].

Statistical analysis

The statistical analysis was conducted using SPSS Software 26.0 version for One-Way ANOVA and Post Hoc Duncan Test. All data were shown in mean ± standard error (SE); a value of $p<0.05$ was considered significant between the two groups.

Results and Discussion

Endometriosis is one of the estrogen-dependent diseases characterized by the existence of endometrial tissue *ex utero*. Previous studies have shown that estrogen with its receptors, namely (ER)-a and ER-b, can enhance NF-κB activity in ectopic endometriotic cells[10]. This estrogen-induced NF-κB activation is mediated by promoting proinflammatory pathways including CXCL12/CXCR4, PI3K/Akt, and thymic stromal lymphopoietin signaling[23]. Moreover, canonical pathway activation of NF-κB as transcription factors in ectopic endometriotic lesions implies the occurrence of aberrant inflammatory responses, which have been previously shown to be highly regulated by p50/p65 NF-κB dimers. These dimers are activated *in vivo* and in response to the activity of TNF-a and IL-1b cytokines in endometrial stromal cells in vitro[10]. NF-κB is also responsible for perpetuating cellular proliferation and growth. In this study, the results showed that the treatment using daily PM extract successfully suppressed the NF-κB activity compared to the control group. This effect was partly consistent with previous studies, where 7.5 and 11.25 mg/kg/day of PM extract were observed to impede NF-κB activity significantly[17]. Generally, PM consists of flavonoids proven to inhibit NF-κB expression under various conditions[24, 25]. One of the main constituents of PM, glycitin, had been shown to antagonize NF-κB activity in intervertebral disc degeneration indirectly[26]. Other compounds, such as naringenin and eriodictyol, were also known to suppress NF-κB activation through MT1G upregulation and TLR4 inhibition[27,28]. Apart from the unidentified cause of action, various factors should be considered such as the dual role of phytoestrogen compounds in modulating inflammatory responses and non-canonical signaling that affect NF-κB upregulation[9,29]. Therefore, this study

provides a basis for proposing an optimum therapeutic dose in the context of endometriosis.

COX-2 level expression

According to the analyses, the induction of endometriosis increased the expression of COX-2, where the ADM group had the highest COX-2 level expression among the other groups ($p<0.05$) (Figure 1). The gradient of COX-2 expression in all groups in response to PM administration differed dose-dependently. Compared to the ADM group, each treatment dose significantly inhibited COX-2 expression ($p<0.05$). The results showed that along with the increase in PM dose treatment, the expression of COX-2 was reduced. Furthermore, 11.25, 15, 18.75, and 22.5 mg/kg/day of PM normalized COX-2 level of endometriosis mice model and the results were not significantly different from the control group. The sustenance of inflammatory response in endometriosis cannot be separated from COX-2 activity, an enzyme that plays a crucial role in converting arachidonic acid into prostaglandin H2. This molecule serves as a precursor of various prostaglandins, including PGE2, which is essential in persisting cell survival, angiogenesis, and resistance to apoptosis[30, 31]. Therefore, upregulation of COX-2 often produces a chronic inflammatory environment and progressive growth of endometriotic lesions[32].

In this study, COX-2 expressions decreased dose-dependently with increasing concentrations of PM extract. The result suggested that the inhibitory capacity of PM to COX-2 was linear to the dose and not reversed by compensatory or positive feedback loop mechanisms. The reduction of COX-2 levels in response to PM might interfere with the activation of PGE2-induced EP2 and EP4, preventing the triggers of cAMP/PKA and PI3K/AKT signaling pathways. By inhibiting these pathways, PM reduces the positive feedback loop that typically sustains COX-2 expression in endometriotic tissues[31, 33].

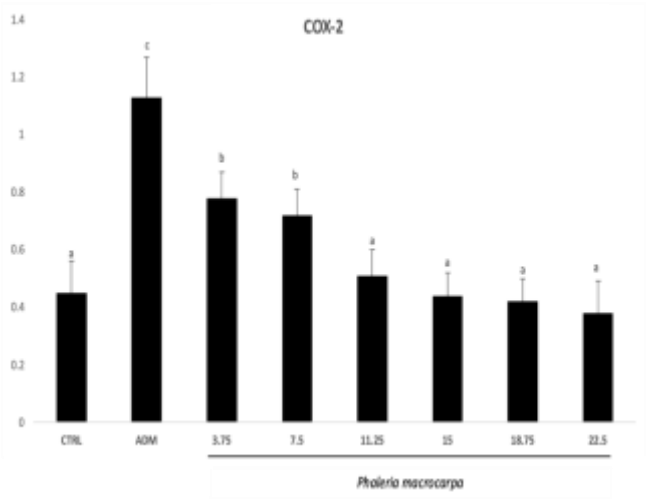

Figure 1: COX-2 level expression in endometriosis mice model. Data are expressed as mean ± SEM (n=4). Treatments not sharing the same letters in the same row are significantly different by ANOVA followed by a Duncan test ($p<0.05$). CTRL; healthy mice control group, ADM; endometriosis mice group, 3.75 – 22.5 PM extract concentration group treated to endometriosis mice.

Immunohistochemistry analysis on the expression of NF-κB p65, VEGFA, and Ki67

This study showed ADM treatment increased NF-κB, VEGF-A, and Ki67 expression significantly compared to the control group ($p<0.05$) (Figure 2A). The expression of NF-κB showed a significant reduction in the group after giving 7.5 mg/kg/day PM extract ($p<0.05$). Specifically, the most significant effect was observed by suppressing the NF-κB expression in the 11.25 mg/kg/day treatment group. The expression of NF-κB in the treatment group of 3.75 and 18.75 mg/kg/day PM extract was not significantly different from the AMD group. In addition, the analysis showed that the level of VEGF-A expression declined after treatment with PM extract at 7.5, 11.25, and 15 mg/kg/day concentration ($p<0.05$) (Figure 2B). However, 3.75 mg/kg/day dose of PM extract could not reduce VEGF-A expression and was not significantly different from the treatment group. The ADM group and 3.75 mg/kg/day PM extract treatment showed a higher level of Ki67 expression (Figure 2). In comparison, the treatment of PM extract with 7.5, 11.25, 15, 18.75, and 22.5 mg/kg/day doses reduced the expression of Ki67 as the control group ($p<0.05$).

Table 1: Immunohistochemistry analysis of NF-κB, VEGF-A, and Ki67 in endometriosis mice

Groups	NF-κB (mean ± SEM)	P value	VEGF-A (mean ± SEM)	P value	Ki67 (mean ± SEM)	P value
CTRL	20.73 ± 0.99^{bc}	<0.05	26.63 ± 2.61^{b}	<0.05	21.49 ± 0.83^{abc}	<0.05
ADM	31.93 ± 1.29^{d}		45.54 ± 4.82^{cd}		33.21 ± 2.26^{cd}	
3.75	32.28 ± 1.97^{d}		47.05 ± 2.7^{d}		39.74 ± 2.45^{d}	
7.5	24.50 ± 3.12^{c}		36.10 ± 6.8^{bc}		18.05 ± 1.62^{ab}	
11.25	12.83 ± 1.2^{a}		13.15 ± 0.84^{a}		21.09 ± 2.45^{abc}	
15	17.51 ± 0.56^{ab}		10.80 ± 0.34^{a}		10.41 ± 0.75^{a}	
18.75	31.79 ± 3.3^{d}		29.36 ± 1.76^{b}		26.10 ± 8.98^{bc}	
22.5	24.47 ± 2.63^{c}		31.75 ± 3.39^{b}		24.83 ± 7.31^{bc}	

The effect of PM in reducing the expression of VEGF-A, a key regulator in angiogenesis was assessed in this study. As the primary formation of new blood vessels, angiogenesis is driven by inflammatory cytokines. It is also essential as nutrient and oxygen supplier for ectopic endometrial tissue survival and growth[34]. Moreover, high expression of VEGF-A sustains a heightened inflammatory milieu by promoting expansion of migrating cells through binding to its receptors on endothelial cells, VEGFR-1 and VEGFR-2. This receptor subsequently triggers the downstream PI3K/Akt and MAPK signalling cascade to enhance more VEGF-A secretion and creates a positive feedback loop that exacerbates the inflammatory response[35]. Therefore, restraining VEGF-A expression becomes an effective way to prevent more endometriotic lesions from growing. Based on the results, PM had significant inhibitory effects at 11.25 and 15 mg/kg/day doses toward VEGF-A expression. Similar to NF-κB, an unexpected increase in VEGF-A level was also observed in the 18.75 and 22.5 mg/kg/day dose groups. The identical pattern showed that VEGF-A was expressed in

the same axis as NF-κB. This was in line with previous *in vivo* and *in silico* studies, showing that one of the main flavonoids in PM, genistein, exerted the ability as an antiangiogenic agent by hindering VEGF expression[22, 36]. Another significant phytoestrogen present in PM, namely naringenin, was known to inhibit VEGF and its downstream effects, as shown by the possible negative regulation towards the AKT signaling pathway[37, 38].

In the endometriotic milieu, enhanced Ki67 expression typically signifies increased cell proliferation within endometriotic lesions. However, the suppression drives apoptosis programming, causing an apoptotic index in most studies on inflammation[39]. This study showed a relatively lower Ki67 expression in response to 7.5-15 mg/kg/day of PM extract. Specifically, 15-dose group exerted the most robust inhibitory effect on Ki67 compared to endometriosis group. Ki67 suppression could lead to weakened endometriotic cell cycle-related signaling, including PI3K/Akt/mTOR pathways, which would cause a decline in endometriotic cell division[40, 41].

Implant area

This study showed that untreated endometriosis mice had the highest implant area (95.06 mm^2) and were significantly different from other groups ($p<0.05$). The treatment of PM extract significantly reduced the area of ADM implant in endometriosis mice, with 7.5 and 15 mg/kg/day dose groups having the smallest value at 49.97 mm^2 and 47.7 mm^2, respectively (Figure 3). The measurement showed that the average implant area of the endometriotic lesion had a similar pattern to Ki67 expression. A more significant reduction in area was observed in 3.75 to 15 mg/kg/day of PM, followed by an increase at doses 18.75 and 22.5 mg/kg/day. This pattern suggested that PM initially suppressed lesion growth, likely through the mechanisms in accordance with Ki67 inhibition-dependent lesion cell apoptosis. Additionally, the reduction in lesion size might be caused by the decrease in COX-2 expression. This was because lowering COX-2 levels would lead to reduced inflammation, a critical factor for the growth and persistence of endometriotic lesions[31]. The increased lesion size at higher doses was considered a probable consequence of efficacy loss affecting the local tissue milieu, which led to compensatory mechanisms and enhanced lesion growth. These mechanisms could include the activation of alternative signaling, such as the JAK/STAT pathway, which might promote cell survival and proliferation during increased stress or damage at higher extract concentrations[32,42].

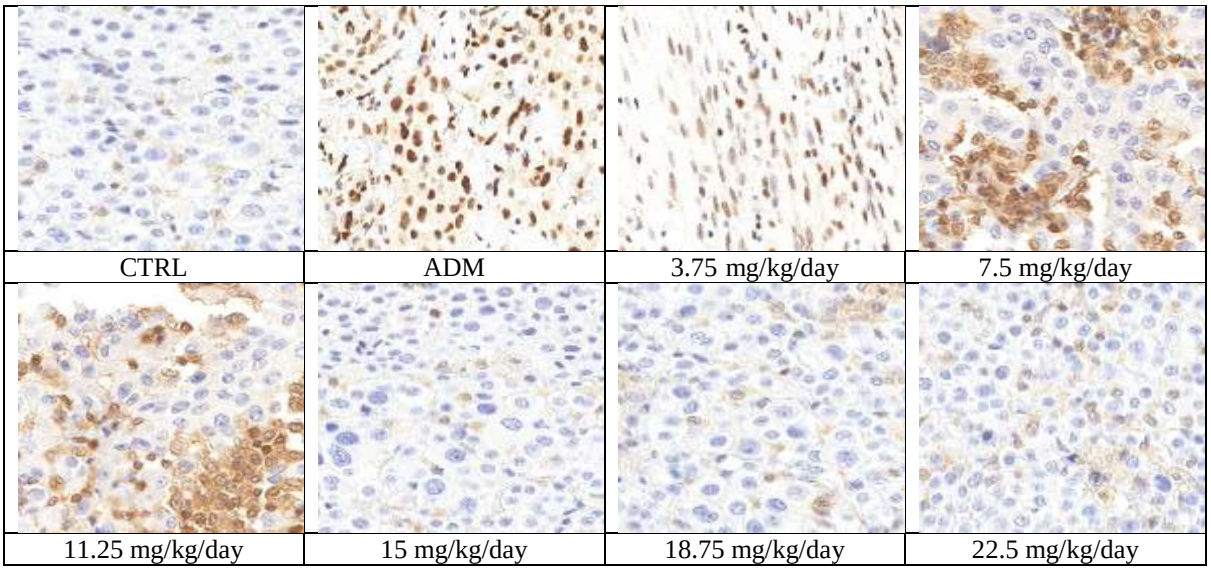

Figure 2: The expression of NfkB p65 in peritoneal tissue (recorded with an AccuView microscope at 400X magnification)

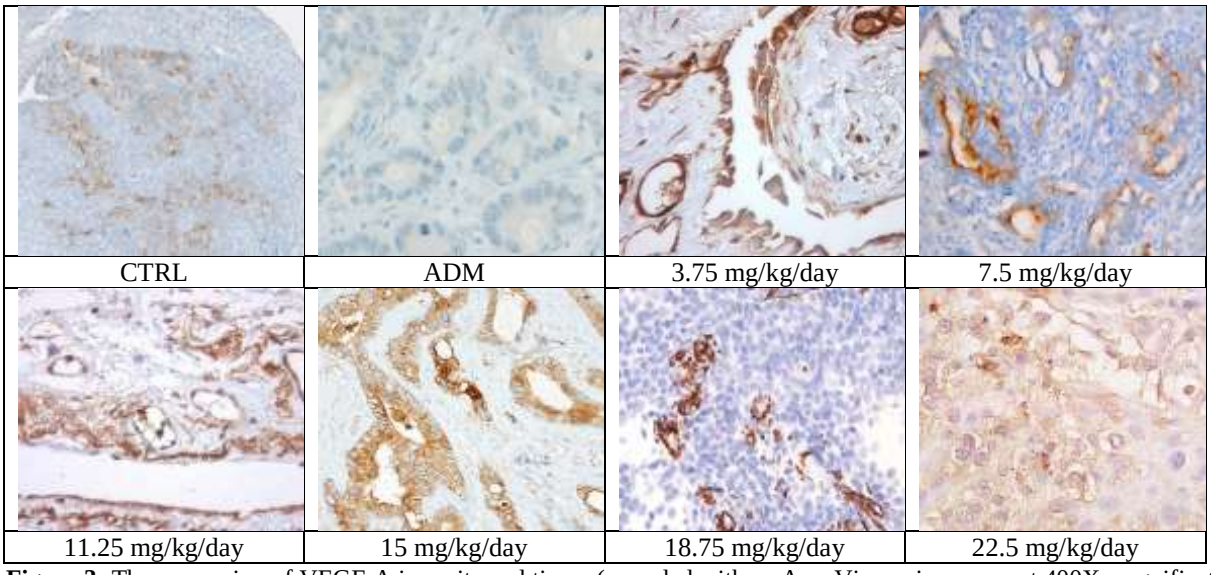

Figure 3: The expression of VEGF-A in peritoneal tissue (recorded with an AccuView microscope at 400X magnification)

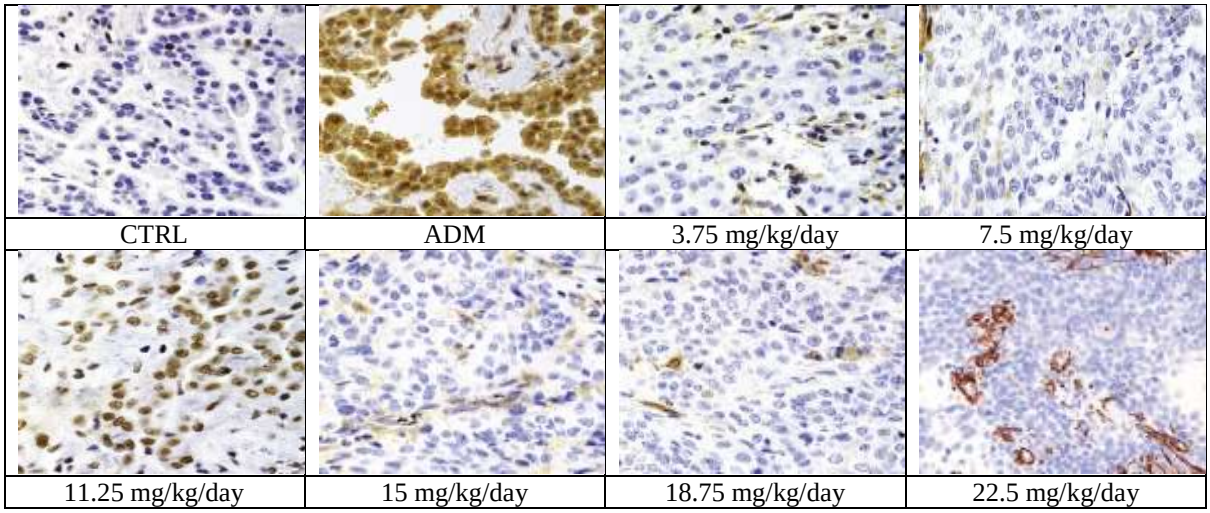

Figure 4: The expression of Ki67 in peritoneal tissue (recorded with an AccuView microscope at 400X magnification)

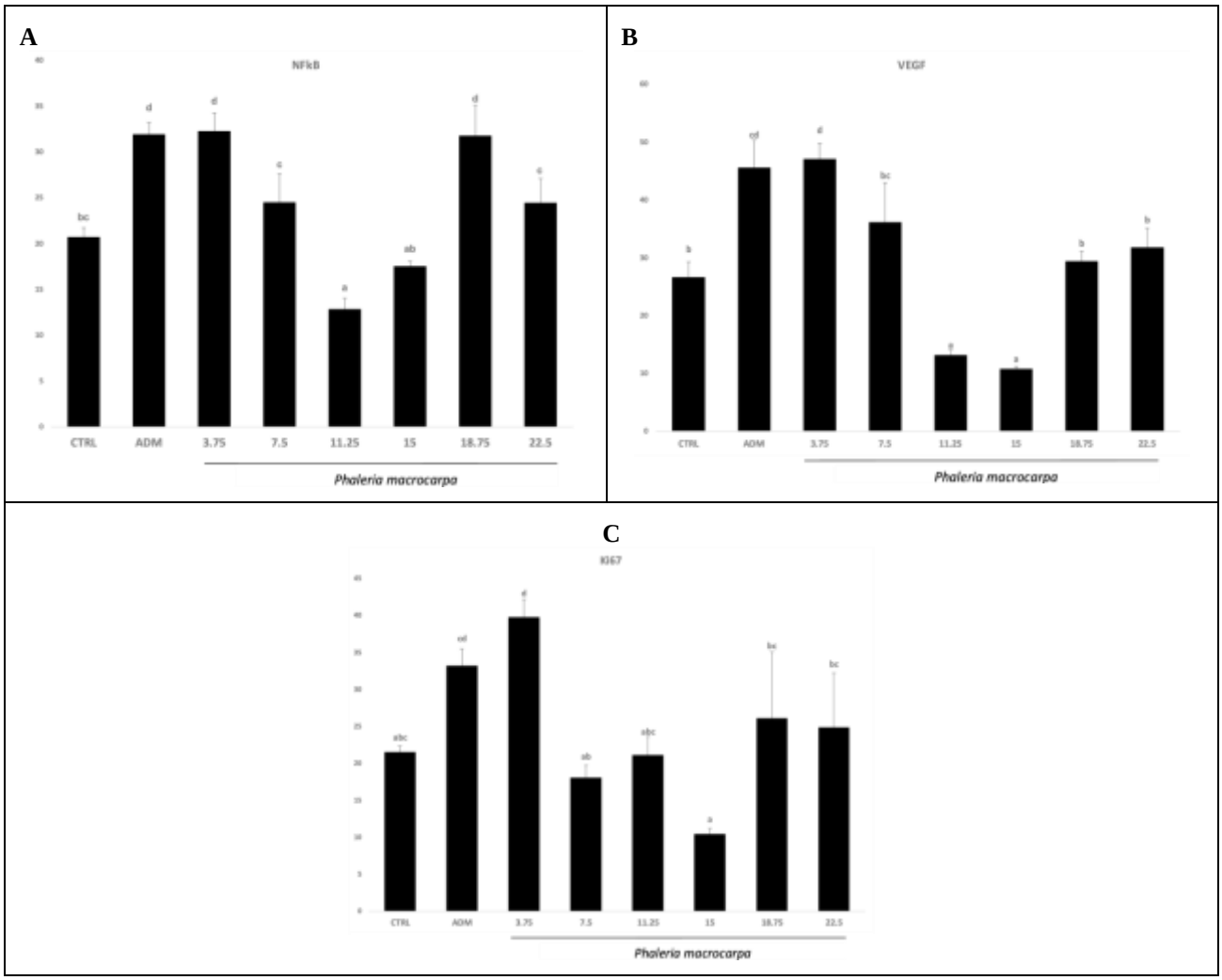

Figure 5: Immunohistochemistry analysis. **(A)** NF-κB p65 expression; **(B)** VEGF-A expression; and **(C)** Ki67 expression. Data are expressed as mean ± SEM (n=4). Treatments not sharing the same letters in the same row are significantly different by ANOVA followed by a Duncan test ($p<0.05$). CTRL; healthy mice control group, ADM; endometriosis mice group, 3.75 – 22.5 PM extract concentration group treated to endometriosis mice.

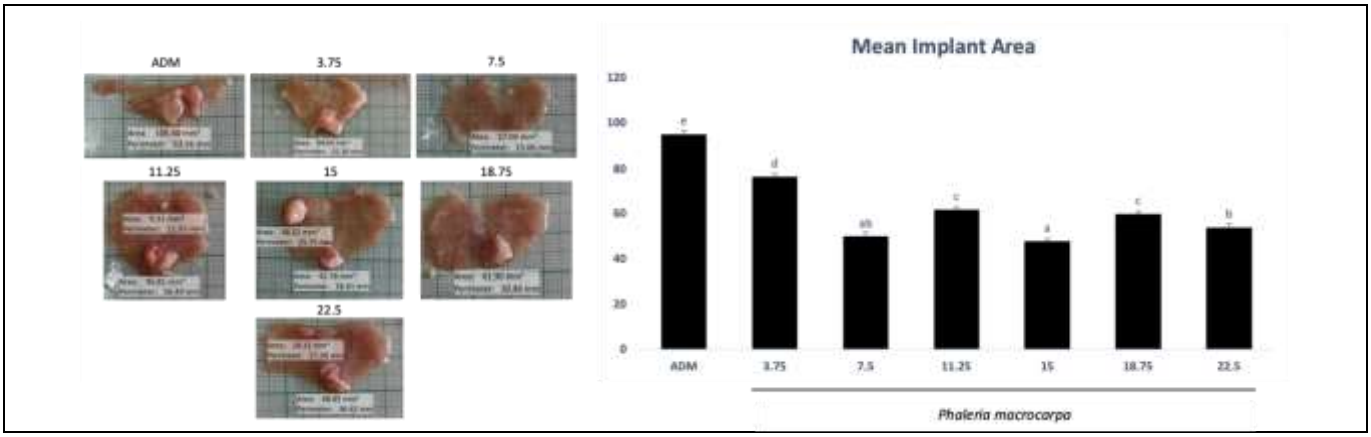

Figure 6: Implant area of endometriosis mice model. Data are expressed as mean ± SEM (n=4). Treatments not sharing the same letters in the same row are significantly different by ANOVA followed by a Duncan test ($p<0.05$). ADM; endometriosis mice group, 3.75 – 22.5 PM extract concentration group treated to endometriosis mice.

This biphasic response in both Ki67 expression and mean implant area shows the importance of selectively optimizing the dose when considering PM as a potential therapeutic agent for endometriosis.

Conclusion

In conclusion, this study showed that PM ethanolic extract could be used for the management of endometriosis. The inhibitory effect was exerted by PM through several mechanisms, such as an anti-inflammatory agent hindering the expression of NFkB and COX-2. Extract also reduced endometriosis lesions and inhibited ADM implantation growth through the inhibition of VEGF-A and Ki67 protein. Moreover, future studies should focus on isolating and characterizing the specific bioactive compounds in the mesocarp responsible for the anti-inflammatory in endometriosis model.

Conflict of Interest

The author`s declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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