Original Article

Red fruit (*Pandanus conoideus*) inhibits the development of endometriosis lesions through downregulation of NF-kB and VEGF expression

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Objective: To investigate the benefits of *P. conoideus* fruit extract on inflammation and angiogenesis modulation on the endometrium of endometriosis animal model.

Methodology: Forty female mice (*Mus musculus*) were divided into two groups (20 in each group). The first group was the endometriosis group. The second group was endometriosis treated with *P. conoideus* fruit extract. Analysis of NF-kB and VEGF expression was carried out by immuno-histochemistry. The analysis of endometriosis lesions was performed histologically.

Results: The expression of NF-kB, VEGF, and area of the endometriosis lesion was significantly reduced due to the administration of *P. conoideus* fruit extract.

Conclusion: *P.conoideus* fruit extract could inhibit angiogenesis and growth of endometriosis lesions through the down regulation of NF-kB activity. This part of the plant can be an alternative herb to inhibit endometriosis progression. (Rawal Med J 202;45:985-989).

Keywords: Endometriosis, peritoneal lesion, inflammation.

INTRODUCTION

Endometriosis is characterized by persistent and developing endometrial tissue in tissues or organs other than the uterus. This has significant functional, psychological, and emotional effects of 5%-10% of fertile women and half of the infertile women. The peritoneal environment associated with an aberrant immune response contributes to increased implantation, proliferation, and angiogenesis.[1-5] Local inflammation in the peritoneal tissue is one of the determinants of endometriosis development.[6] The body's immune response is controlled regularly and complexly, one of which is through the activation of NF-kB. NF-kB also controls proliferation, apoptosis, adhesion, invasion, and angiogenesis.[7] The NF-kB expression is higher in endometriosis lesions.[8] In endometriosis, pro-inflammatory cytokines as one of the NF-kB products can be stimulated by IL-10 and TNF-a.[9] In endometriosis lesions, the relationship between NF-kB activity and VEGF-A indicates an increase in angiogenesis.[10-12] Therefore, the inhibition of the action of NF-kB can certainly suppress the molecular pathology associated with endometriosis.

Pandanus conoideus (P. conoideus), locally know as red fruit, is a native plant in Indonesia, thrives in the forests of the island of Papua. Indigenous people use this oil-rich fruit as a functional food.[13] Several studies have revealed the benefits of red fruit in modulating inflammatory diseases. However, some of the plant's pharmacological effects are inconsistent. This plant can also stimulate the free radical formation and trigger oxidative stress.[14-16] For atherosclerosis inhibition, there is an effect of decreasing foam cells at certain doses.[17] The capacity of *P. conoideus* in suppressing the action of NF-kB certainly also suppresses its inflammatory cytokine products (TNF-a and IL-10).[18,19] To our knowledge, there has been no study that uses *P. conoideus* fruit extract for endometriosis. Therefore, this study aimed to investigate the anti-endometriosis effects of *P. conoideus* on the modulation of inflammation and angiogenesis as critical molecular events in endometriosis.

METHODOLOGY

A total of 40 female mice (*Mus musculus*), 12 weeks old, bodyweight 20-30 g, were divided into two groups (20 in each group), namely the endometriosis group (EN) and endometriosis group plus *P. conoideus* fruit extract (ENPC). The dosage

of *P. conoideus* fruit extract was 0.05 ml/day for 28 days. The extract was given by oral lavage. Mice were kept individually in a standard cage (temperature 25 ± 2°C with 12 hours dark and light cycle). They were allowed to access food and drink ad libitum. Before being given the treatment, the animals were acclimatized for one week.

P. conoideus extract was obtained as syrup from the commercial market (Planta Sehat Sari Buah Merah Brand, CV made Mulya Asli, Jayapura, Papua, Indonesia). The LC-HRMS techniques was used to identify the phytochemicals of *P. conoideus*. High-Performance Liquid Chromatography fingerprint analysis was carried out at 25 ± 1°C. The extract was dissolved in acetonitrile and filtered according to standard procedures. The sample is then fed into the autosampler and then injected into the device. The analysis was carried out by high performance liquid chromatography (Thermo Scientific Dionex Ultimate 3000 RSLC Nano with a microflow meter). The mobile phase was 0.1% formic acid in water or acetonitrile. The flow rate was set at 40 mL/min; for 30 min and a column (Hypersil GOLD aQ 50 x 1.9 mm x 1.0 mm particle size) temperature of 30°C. HR-MS analysis was performed using Thermo Scientific Q Exactive tools. The full scan was done at standard conditions. For data processing, we use the Compound Discoverer software in mzCloud MS/MS Library.

After the treatment was complete, mice were euthanized and surgically removed to isolate the peritoneal tissue. Before surgery, mice received ketamine and xylazine as an anesthetic agent. Peritoneal tissue samples were stored at −80°C until analysis.

Analysis of NF-kB and VEGF expression from reddish lesions in the peritoneal tissue was carried out by immunohistochemistry (IHC) techniques. The analysis was performed according modification in the previous procedure. IHC analysis was determined by multiplying the percentage score of immunoreactive cells with the color intensity score that emerged from the immunoreactive cells. The results are the mean of the five fields of view. Results are expressed on an immunoreactive scale (IRS scale). The magnification used to be 400x. The examination was done using a Nikon H600L light microscope.

IHC staining using a 3.3'-diaminobenzidine stain kit (DAB) (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) with the counterstaining using Coomassie Briliant Blue (Sigma Aldrich, Merck KGaA, Darmstadt, Germany) was performed. NF-kB p65 antibody (Santa Cruz Biotechnology, Catalog number sc-515045) and VEGF antibody (Santa Cruz Biotechnology, Catalog number sc-7269) was used as primary antibody. The area of endometriosis implants was assessed microscopically in areas with hyperemia and hypervascularization. The number of reddish lesions and hypervascularizing lesions was assumed to be the area of implant endometriosis. The calculation was performed using computerized analysis (Motic software, Kowloon, Hongkong). The Research Ethics Committee, Faculty of Medicine, Airlangga University, Surabaya, East Java, Indonesia was approved this study (No. 388-KE). All procedures were performed according to guidelines and regulations of animal welfare.

Statistical Analysis: Statistical analysis was performed using SPSS version 20. Expressions have been represented as mean ± standard of deviation. The independent t-test was performed to detect the differences between groups.

RESULTS

Table displays the LC-HRMS results from *P. conoideus* fruit extract. There are four possible of active compounds, including oleamide, palmitoleic acid, eicosatetraynoic acid, and methyl palmitate, as shown in (Figure 1).

Table. Composition of phytocomponent in the P. Conoideus.

Peak	Retention time	Name of compound	Formula	Molecular weight
1	17.039	Oleamide	C18H35NO	281.2707
2	16.039	Palmitoleic acid	C16H30O2	254.2235
3	14.017	Eicosatetraynoic acid	C20H24O2	308.1951
4	10.634	Methyl palmitate	C17H34O2	287.2811

Fig 1. Active compounds.

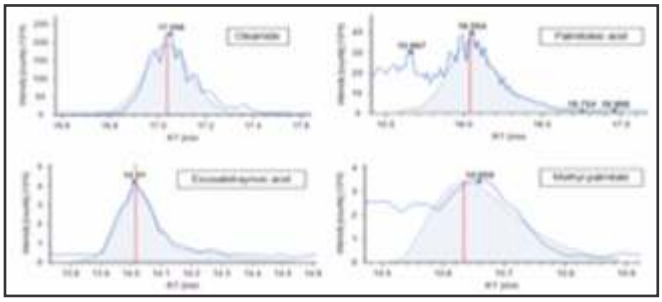
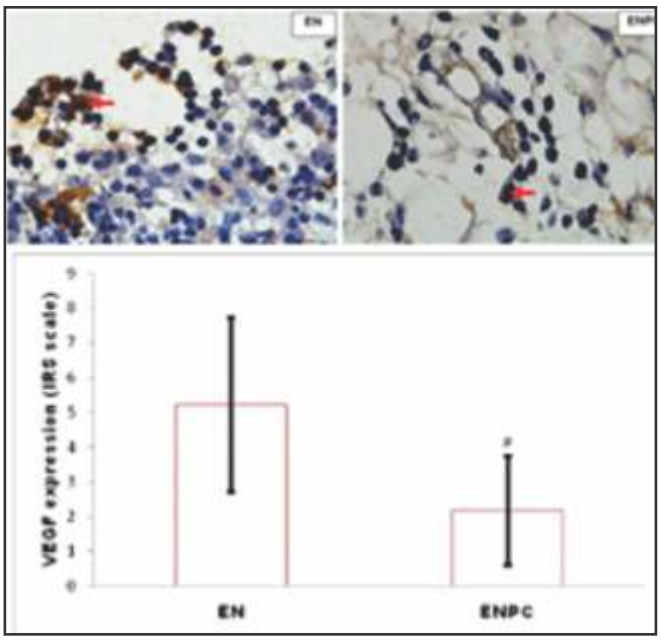


Fig 2. NF-kB expression.



NF-kB expression in EN and ENPC group can be seen in Fig. 2. There was a significant down-regulation (3.56-fold) in NF-kB expression in the ENPC group (5.34±2.14 IRS sclae) compared to the control group (1.50±1.40 IRS sclae) ($p<0.05$). While VEGF peritoneum expression significantly down-regulation (2.39-fold) in the ENPC group (5.23±2.52 IRS sclae) compared to the EN group (2.18±1.57 IRS sclae) ($p<0.05$). The implant area in the ENRF group was significantly smaller (3.34-fold) in the group that was given extracts (70.45±17.46 mm^2) compared to the EN group (21.05±11.14 mm^2) ($p<0.05$).

DISCUSSION

Our study reveals that red fruit can reduce NF-kB expression. This shows that red fruit can inhibit inflammation through modulation of NF-kB activation in endometriosis. We suspect that various active compounds of *P. conoideus* fruit extract can block the translocation of NF-kB. Previous studies explain that methyl palmitate can inhibit NF-kB expression.[20] Methyl palmitate can activate PPAR-g so that it downregulates the NF-kB pathway.[21] Besides, methyl palmitate is also a phosphorylation inhibitor of IkB.[22] Eicosatetraenoic acid inhibits the translocation of NF-kB to the nucleus.[23,24] In this study, the decrease in NF-kB expression was followed by a reduction of VEGF expression and the area of endometriosis lesions. These findings indicate that the active compound of *P. conoideus* fruit extract is able to suppress the activation of NF-kB so that the production of VEGF as an angiogenesis bio factor is also suppressed. Furthermore, this inhibition will impair lesion growth, as evidenced in this study.

We found that in this study had non-opaque red and opaque red lesions. A limitation of this study is the absence of a comparison of various doses. This will be the focus of future research. In addition, four active compounds that are candidates for anti-endometriosis are also a concern in future studies.

CONCLUSION

Various active compounds from *P. conoideus* fruit extract could inhibit angiogenesis and growth of endometriosis lesions through downregulation of NF-kB activity. Thus, *P. conoideus* fruit extract can be an herb or food supplement to inhibit development of endometriosis.

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