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# The Impact of *Phaleria macrocarpa* Fruit Flavonoid Extract on Vascular Endothelial Growth Factor (VEGF) Expression in the Endometrium and Myometrium of Menopausal Mice Model

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**Abstract:** Menopause represents a critical transitional phase in a woman's life, signifying the cessation of menstrual cycles due to declining ovarian function and reduced estrogen production. This study investigates the effects of Phaleria macrocarpa fruit flavonoid extract on Vascular Endothelial Growth Factor (VEGF) expression in the endometrium and myometrium of menopausal mouse models, focusing on its potential to mitigate hormonal imbalances and associated vascular changes. The experimental design involved 32 mice divided into a negative control group, a positive control group (ovariectomized and untreated), and four treatment groups receiving varying doses of Phaleria macrocarpa extract. Administration was oral via gastric gavage for 14 days post-ovariectomy. VEGF expression was evaluated using immunofluorescence staining and quantified using ImageJ software. Results demonstrated that higher doses of Phaleria macrocarpa flavonoid extract (7.5 mg/mouse/day, 11.25 mg/mouse/day, and 15 mg/mouse/day) significantly decreased VEGF expression in both the endometrium (p-values: P2 0.013, P3 0.010, P4 0.009) and myometrium (p-values: P1 0.001, P2 0.000, P3 0.000, P4 0.000) compared to the positive control group. Pearson correlation analysis indicated a strong negative correlation (r = -0.600 in endometrium, r = -0.637 in myometrium) between Phaleria macrocarpa flavonoid extract doses and VEGF expression, highlighting a dose-dependent effect on vascularization. This study underscores the potential of Phaleria macrocarpa flavonoid extract as an alternative therapy to modulate VEGF expression in menopausal tissues, offering insights into its therapeutic efficacy and contributing to the development of novel treatments for menopausal health issues.

**Keyword:** Phaleria Macrocarpa, Menopause, VEGF Expression, Endometrium, Myometrium

## INTRODUCTION

Menopause is the transitional period from the reproductive to the non-reproductive phase, marked by the cessation of menstrual cycles. It is an inevitable part of aging for women, typically occurring between the ages of 45 and 55, with Indonesian women experiencing menopause at an average age of 50. As women age, ovarian function declines, leading to decreased estrogen production and subsequent menopause. This transition brings about various physical and psychological changes (Suparni & Astutik, 2016). Approximately 75% of women perceive menopause as a significant issue, whereas 55% do not consider it problematic (Asbar, 2018). Menopause induces numerous physiological changes in a woman's body, particularly in the endometrium and myometrium, which are uterine layers influenced by hormonal fluctuations. The reduction in estrogen levels during menopause leads to structural and functional alterations in these tissues, including decreased vascularization.

These changes are often accompanied by various health disorders and symptoms that affect women's quality of life, such as osteoporosis, cardiovascular diseases, sleep disturbances, mood disorders, and reproductive health issues. Reduced levels of Vascular Endothelial Growth Factor (VEGF) during menopause result in diminished angiogenesis, leading to endometrial or vaginal atrophy, which can cause uterine bleeding (Johnson, 2022). According to the World Health Organization (WHO), women's life expectancy globally has been increasing annually, reaching 74.2 years in 2019 (WHO, 2019).

VEGF plays a crucial role in maintaining the health of these tissues through angiogenesis. Estrogen is essential for angiogenesis and the activation of angiogenic factors like VEGF, which is critical for endometrial proliferation during the menstrual cycle (Nayak & Brenner, 2002). During the proliferative phase, angiogenesis occurs, involving the formation and growth of new blood vessel branches, including arterioles, which regress after menstruation. Suboptimal estrogen secretion can impede endometrial blood vessel formation (Fritz & Speroff, 2011).

VEGF is vital for new blood vessel formation and maintaining the integrity and continuity of the endometrium and myometrium. Previous studies have shown that VEGF expression decreases during menopause, contributing to reduced vascularization in these tissues. Inadequate blood supply to the endometrium and myometrium can adversely affect tissue health and contribute to menopausal health issues.First-line therapy for menopausal women with genitourinary symptoms typically targets symptomatic relief. Hormonal therapies, while effective, require prolonged treatment and may have side effects like endometrial hyperplasia, breast cancer, colorectal cancer, and ovarian cancer. Alternative therapies are being developed to address menopausal effects on the vagina, endometrium, and myometrium, including the use of flavonoids as phytoestrogen alternatives (Andini et aI., 2023).

Phytoestrogens are plant-derived compounds structurally similar to 17β-estradiol, classified into isoflavones, stilbenes, coumestans, and lignans. Isoflavones, a type of flavonoid, contain hydroxyl groups necessary for estrogenic activity. They can serve as estrogen substitutes by binding to estrogen receptors, forming active hormone-receptor complexes that interact with DNA, potentially increasing uterine vascularization and endometrial thickness (Ariyanti & Apriliana, 2016). Flavonoids are natural compounds found in plants with potential health benefits. Extracts of Phaleria macrocarpa (family Thymelaeaceae) contain active components like alkaloids, saponins, flavonoids, and polyphenols. Studies have shown that Phaleria macrocarpa

extract possesses antioxidant, anti-inflammatory, and estrogenic effects. However, research on the impact of Phaleria macrocarpa flavonoid extract on VEGF expression and arteriole ratio in the endometrium and myometrium of menopausal models is limited.

This study aims to investigate the effects of Phaleria macrocarpa flavonoid extract on VEGF expression and arteriole count in the endometrium and myometrium of menopausal mice (Rattus norvegicus). Previous research by Liem et al. (2019) demonstrated that flavonoids in Phaleria macrocarpa extract could enhance vascularization in rat muscle tissue. Susanto et al. (2021) found that Phaleria macrocarpa extract exhibited strong antioxidant effects. This study's novelty lies in using Phaleria macrocarpa flavonoid extract on menopausal mice models to assess its impact on VEGF expression in the endometrium and myometrium, aiming to improve histopathological profiles. This research seeks to provide scientific evidence supporting the beneficial effects of Phaleria macrocarpa flavonoid extract on VEGF expression ratio in menopausal endometrium and myometrium.

## MATERIALS AND METHOD

### Study Design

This study was using a true experimental research design with a post-test only control group approach, has been undergone an ethical clearance by the Health Research Ethics Committee, Faculty of Medicine, Brawijaya University, and has been obtained the Ethical Clearance Certificate with the approval code No. 35/EC/KEPK-S2/01/2024.

### Materials

A total of 32 mice, aged 6-8 weeks and weighing between 20-30 grams, in healthy and fit condition, were utilized as experimental animals in this study. The mice underwent a 7-day acclimatization period before being divided into two groups: a negative control group and a positive control group, prior to undergoing ovariectomy. The ovariectomy procedure was performed under anesthesia, using a combination of 80 mg/kg body weight ketamine and 10 mg/kg body weight xylazine. After 28 days, it was confirmed that the mice in the treatment groups had reached menopause, as indicated by elevated FSH levels in one of the mice. Subsequently, the mice were divided into six groups: one negative control group (not ovariectomized), one positive control group (ovariectomized and not treated), and four treatment groups (P1: OVX and administered 3.75 mg/mouse/day extract; P2: OVX and administered 7.5 mg/mouse/day extract; P3: OVX and administered 11.25 mg/mouse/day extract; P4: OVX and administered 15 mg/mouse/day extract). The extract was administered orally for 14 days using a gastric gavage. (Maharani & Sutrisno, 2021).

### Flavonoid Extract Preparation

Flavonoid extraction from the fruit of Phaleria macrocarpa was performed using an herbal extraction technique with 96% ethanol as the solvent. The use of 96% ethanol, being semi-polar, results in a higher yield, which may be attributed to the predominantly non-polar nature of the contained flavonoids, thereby enhancing flavonoid extraction (Pujiastuti and El'Zeba, 2021; Maharani and Sutrisno, 2022).

In this study, mature, maroon-red fruits were selected, and their flesh and skins were separated after the seeds were removed. The cleaned fruits were then dried. A total of 2500

grams of powdered Phaleria macrocarpa was obtained from the processed sample. This powder was immersed in 30L of 96% ethanol, with continuous stirring for approximately 30 minutes to ensure thorough mixing.

The mixture was left to stand for 5 days to allow sedimentation, with constant stirring throughout the process. The resultant solution was then filtered using a Büchner funnel to obtain the macerate. The macerate, still containing ethanol, was subjected to evaporation for 8 hours at 60˚C to yield a concentrated extract. Subsequently, the flavonoid compounds were separated via liquid-liquid partitioning using n-hexane to achieve homogeneity. The mixture was left undisturbed until phase separation occurred between the aqueous and n-hexane fractions. The separation of the aqueous fraction from the n-hexane fraction was repeated three times. Following the precipitation, the n-hexane layer was evaporated at approximately 45˚C. Finally, the flavonoid compounds were further separated using n-butanol partitioning.

### VEGF Expression Analysis

The uterine organs of mice were obtained through transverse peritoneal surgery under the influence of 0.2 ml of 1% ketamine anesthesia. The uterine specimens were then preserved in a 10% buffered formalin solution. Histopathological preparations involved longitudinal sectioning of the uterus into approximately 2-3 mm thick slices, followed by processing using a Thermo Scientific STP 120 tissue processor for 6 hours. This was followed by paraffin blocking and deparaffinization, and subsequent Immunofluorescence (IF) staining using VEGF C-1 FITC antibody (sc-7269), concluding with mounting using Entellan and covering with a coverslip. VEGF expression was observed using an Olympus microscope at 400x magnification, and VEGF expression quantified using ImageJ software.

## RESULTS AND DISCUSSION

### FSH Levels Concentration

FSH examination was conducted on day 28 post-OVX to confirm the menopausal status of the mice. Random selection of 2 mice was performed prior to grouping into treatment groups. Results of FSH levels were obtained from the control group mice that did not undergo ovariectomy and were not administered Phaleria macrocarpa fruit extract, as well as representative samples from all treatment groups that underwent ovariectomy and received Phaleria macrocarpa fruit extract.

**Table 1. The Mean of FSH Levels in Mice (Mus musculus)**

| No. | Observation Group | Mean FSH Level (IU/L) | Standard Deviation (SD) |
|---|---|---|---|
| 1. | K- | 1,30 | 0,40 |
| 2. | K+ | 6,19 | 0,19 |

Note: Measurement of FSH levels in the blood of mice in the negative control group (KN; no ovariectomy performed) and positive control group (KP; ovariectomy performed) with treatment administered over 28 days; SD: Standard Deviation.

Table 5.1 illustrates that the average FSH levels in the positive control group (K+), which underwent ovariectomy, increased to 6.19 IU/I compared to the negative control group (K-) that did not undergo ovariectomy, with a level of 1.30 IU/I. Based on this increase in average levels, the positive control group is confirmed to be in a menopausal state.

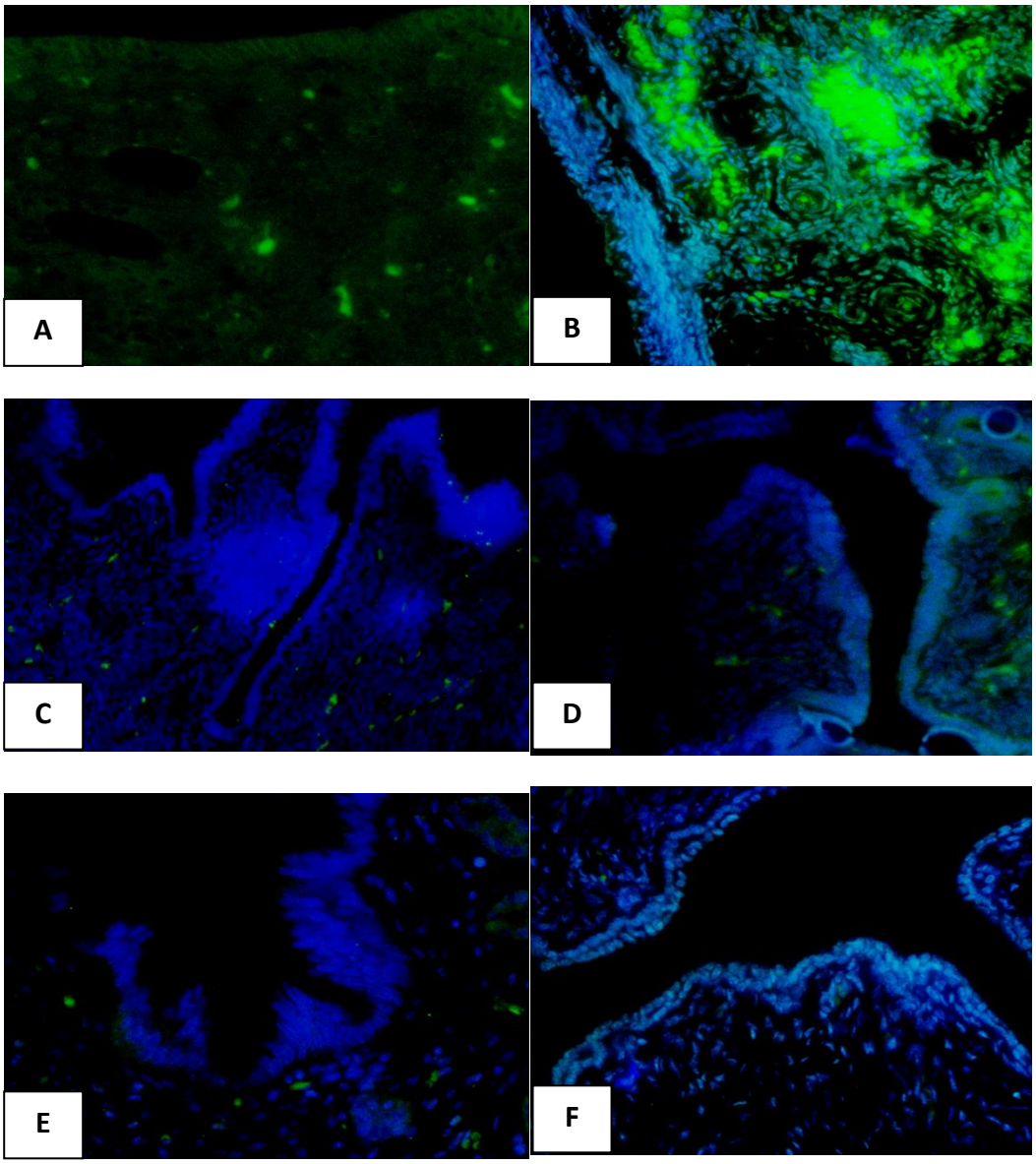


Figure 1. VEGF Expression on Mice Endometrium Endothelial Cells at 400x magnification.

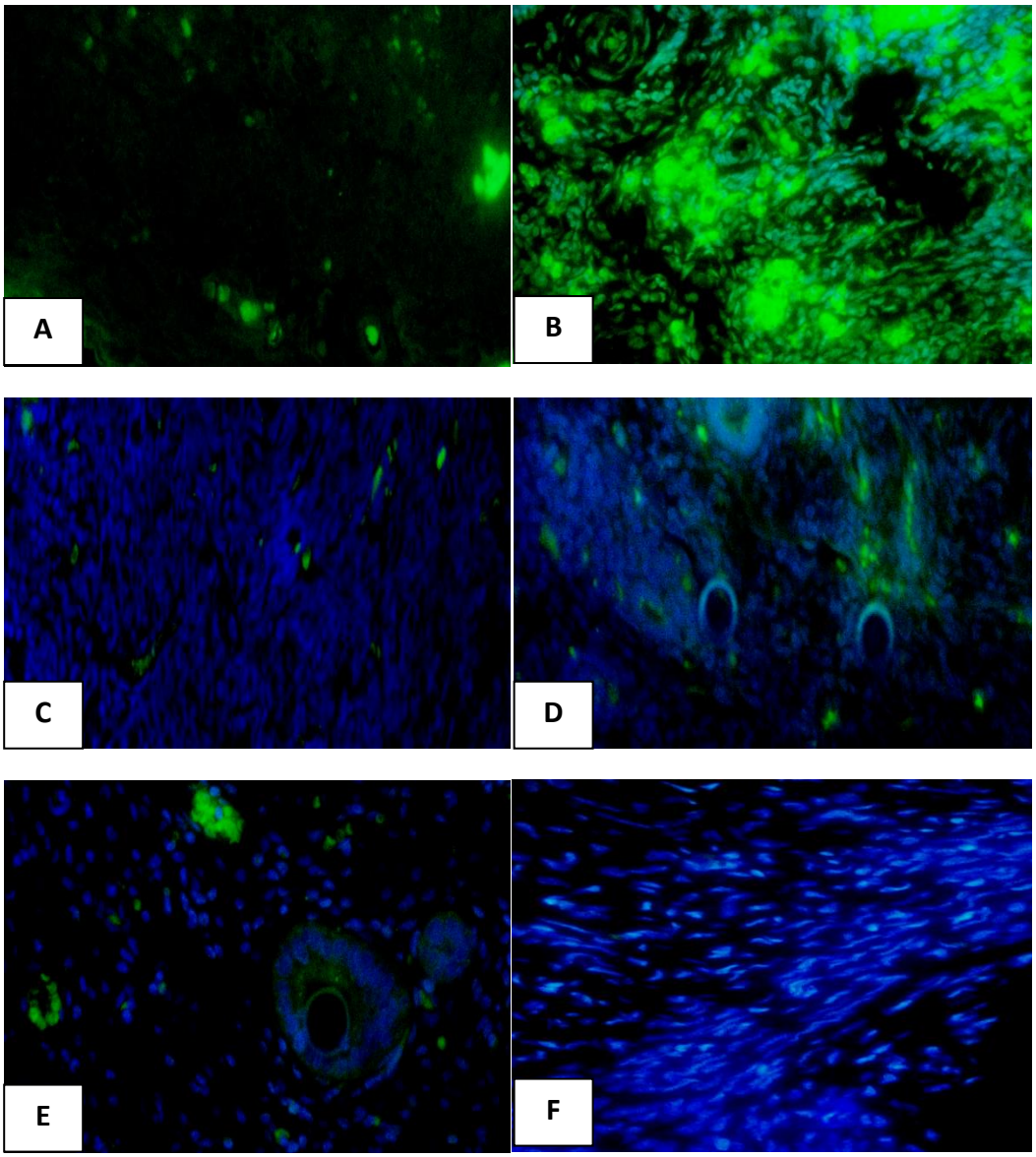


Figure 2. VEGF Expression on Mice Myometrium at 400x magnification.

**Table 2. The Result of One-Way Anova Test on VEGF Expression in the Endometrium and Myometrium of Mice Menopausal Model**

| No. | Observation Group | Mean VEGF ng/l ± SD | | *p-value* | |
|---|---|---|---|---|---|
| | | **Endometrium** | **Myometrium** | **Endometrium** | **Myometrium** |
| 1. | K- | 13450,25±18440,43 | 7114.82±8157,49 | 0,003 | 0.000 |
| 2. | K+ | 75069,44±15681,89 | 78969.69±17508,84 | | |
| 3. | T1 | 34164,20±29936,11 | 19107.08±23972,58 | | |

| | | | |
|---|---|---|---|
| 4. | T2 | 18999,96±28301,00 | 14723.15±27150,23 |
| 5. | T3 | 17720,23±17390,75 | 11296.56±13519,81 |
| 6. | T4 | 16458,25±30888,04 | 10540.94±30912,86 |

Note: If the p-value is < 0.05, it indicates a significanr difference, whereas if the p-value is > 0.05, it indicates no significant difference.

The study reveals that in the positive control group, consisting of ovariectomized mice without flavonoid extract treatment, there was an increase in VEGF expression in both the endometrium and myometrium compared to the negative control group, comprising mice receiving no treatment, with values of 13450.25±18440.43 in the endometrium and 7114.82±8157.49 in the myometrium. Conversely, in the positive treatment group administered with *Phaleria macrocarpa* fruit flavonoid extract, a decrease in VEGF expression was observed in both the endometrium and myometrium compared to the positive control group of menopausal mice not given *Phaleria macrocarpa* flavonoid extract.

The average VEGF expression values in treatment group 1 (T1) were 34164.20±29936.11 in the endometrium and 19107.08±23972.58 in the myometrium with a dose of 3.75 mg/mouse/day, in treatment group 2 (T2) the VEGF expression values were 18999.96±28301.00 in the endometrium and 14723.15±27150.23 in the myometrium with a dose of 7.5 mg/mouse/day, in treatment group 3 (T3) the values were 17720.23±17390.75 in the endometrium and 11296.56±13519.81 in the myometrium with a dose of 11.25 mg/mouse/day, and in treatment group 4 (T4) the values were 16458.25±30888.04 in the endometrium and 10540.94±30912.86 in the myometrium with a dose of 15 mg/mouse/day, showing a decrease compared to the positive control group.

The One Way ANOVA test results yielded a p-value of 0.003 for the endometrium and 0.000 for the myometrium, both smaller than α = 0.05, indicating a significant effect of *Phaleria macrocarpa* fruit flavonoid extract at different doses on VEGF expression in the endometrium and myometrium of menopausal Mus musculus models over a period of 14 days. However, this analysis did not specify which groups exhibited significant differences.

**Table 3. The Result of Tukey Honestly Significant Difference (HSD) on VEGF Expression in the Endometrium of Mice Menopausal Model**

| ***p-value*** | **KN** | **KP** | **T1** | **T2** | **T3** | **T4** |
|---|---|---|---|---|---|---|
| **K-** | | 0,005* | 0,746 | 0,999 | 1,000 | 1,000 |
| **K+** | | | 0,113 | 0,013* | 0,010* | 0,009* |
| **T1** | | | | 0,913 | 1,000 | 1,000 |
| **T2** | | | | | 1,000 | 1,000 |
| **T3** | | | | | | 1,000 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **T4** | | | | | | |

The table 3 shows the results of the Tukey HSD test among different groups. The positive control group (K+) significantly differed from the negative control group (K-) with a p-value of 0.005 ($p<0.05$), indicating that ovariectomy in the K+ group increased Vascular Endothelial Growth Factor (VEGF) expression in mouse endometrium compared to KN. Treatment Group 1 (T1) did not differ significantly from NC or PC, suggesting that administering 3.75 mg/mouse/day of flavonoid did not reduce VEGF expression significantly.

Treatment Group 2 (T2) showed significant differences compared to PC with a p-value of 0.013 ($p<0.05$), but not compared to NC or T1, although a decrease in VEGF expression was observed. Treatment Group 3 (T3) and Treatment Group 4 (T4) also differed significantly from PC with p-values of 0.010 ($p<0.05$) and 0.009 ($p<0.05$), respectively, but did not differ significantly from NC, T1, T2, or T3, showing a reduction in VEGF expression in the menopausal mouse endometrium model. These results indicate that flavonoid extract from Phaleria macrocarpa significantly reduced VEGF expression in the menopausal mouse endometrium model at doses of 7.5 mg/mouse/day, 11.25 mg/mouse/day, and 15 mg/mouse/day.

**Table 4. The Result of Tukey Honestly Significant Difference (HSD) on VEGF Expression in the Myometrium of Mice Menopausal Model**

| ***p-value*** | **KN** | **KP** | **T1** | **T2** | **T3** | **T4** |
|---|---|---|---|---|---|---|
| **KN** | | 0,000* | 0,919 | 0,988 | 0,999 | 1,000 |
| **KP** | | | 0,001* | 0,000* | 0,000* | 0,000* |
| **T1** | | | | 0,999 | 0,987 | 0,980 |
| **T2** | | | | | 1,000 | 0,999 |
| **T3** | | | | | | 1,000 |
| **T4** | | | | | | |

The results of Tukey's HSD test above shows that each group showed significant differences. In the positive control group (K+), there was a significant difference compared to the negative control group (K-) with a p-value of 0.000 ($p < 0.05$), indicating that ovariectomy in the positive control group (K+) increased VEGF expression in the mice myometrium compared to the negative control group (K-).

In contrast, Treatment Group 1 (T1) did not show significant differences compared to the negative control group (K-) with a p-value of 0.919 ($p > 0.05$), but a significant difference was found compared to the positive control group (K+) with a p-value of 0.001 ($p < 0.05$). Treatment Group 2 (T2) showed a significant difference with the positive control group (K+) with a p-value of 0.000 ($p < 0.05$), but there were no significant differences compared to the negative control group (K-) and Treatment Group 1 (T1), although a decrease in VEGF expression was observed.

This pattern was also observed in Treatment Groups 3 (T3) and 4 (T4), which showed significant differences compared to the positive control group (K+) with a p-value of 0.000 ($p < 0.05$). However, there were no significant differences compared to the negative control group or Treatment Groups 1, 2, and 3 ($p > 0.05$), although a decrease in VEGF expression was evident in the myometrium of menopausal mouse models. These findings indicate that the administration of Phaleria macrocarpa fruit flavonoid extract significantly reduced VEGF expression in the myometrium of menopausal mouse models at all variation of doses (3.75 mg/mouse/day, 7.5 mg/mouse/day, 11.25 mg/mouse/day, and 15 mg/mouse/day).

**Table 5. The Result of Pearson's Correlation Test**

| Variable | Correlation Coefficient | Direction of Correlation | Interpretation | *P-Value* |
|---|---|---|---|---|
| Dose of Phaleria macrocarpa Flavonoid Extract on VEGF Expression in Endometrium | 0,600 | Negative | Increasing doses of Phaleria macrocarpa flavonoid extract have an inverse effect, decreasing VEGF expression in menopausal mouse endometrium. | (0,002)** |
| Dose of Phaleria macrocarpa Flavonoid Extract on VEGF Expression in Myometrium | 0,637 | Negative | Increasing doses of Phaleria macrocarpa flavonoid extract have an inverse effect, decreasing VEGF expression in menopausal mouse myometrium. | (0,001)** |

In the table 5, it was stated that the results of the Pearson's correlation test showed a significant relationship or correlation between the variable doses of Phaleria macrocarpa fruit flavonoid extract and VEGF expression in the endometrium and myometrium of the menopausal mouse model (p-value 0.001; $r = 0.600$ in the endometrium; $r = 0.637$ in the myometrium). The correlation coefficients, $r = 0.600$ and $r = 0.637$ respectively, with a negative direction of correlation (-), indicate a strong inverse relationship. This suggests that higher doses of Phaleria macrocarpa fruit flavonoid extract lead to decreased VEGF expression in both the endometrium and myometrium.

Based on the results of One-Way ANOVA and Tukey's Honestly Significant Difference (HSD) test, significant differences were found in the average VEGF expression levels in the

endometrium and myometrium of menopausal mouse models treated with different doses of flavonoid extract from Phaleria macrocarpa. The administration of Phaleria macrocarpa flavonoid extract at doses of 7.5 mg/mouse/day, 11.25 mg/mouse/day, and 15 mg/mouse/day significantly decreased VEGF expression in the endometrium (P2 0.013; P3 0.010; P4 0.009) and myometrium (P1 0.001; P2 0.000; P3 0.000; P4 0.000), with p-values < 0.05. Table 5.7 indicates that Pearson correlation analysis demonstrated a significant relationship between the doses of Phaleria macrocarpa flavonoid extract and VEGF expression in the endometrium and myometrium of menopausal mice (p-value 0.001; r = 0.600 in endometrium; r = 0.637 in myometrium). A correlation coefficient of r = 0.600 and r = 0.637 with a negative correlation direction (-) suggests a strong inverse relationship, indicating that higher doses of Phaleria macrocarpa flavonoid extract lead to decreased VEGF expression in the endometrium and myometrium. This finding aligns with previous research by Eihady et al. (2020), which demonstrated that flavonoid administration reduces VEGF by inhibiting HIF-α transcriptional activity. Isoflavones, such as genistein, a major component of flavonoids, exert potent anti-inflammatory effects and act as phytoestrogens. Phytoestrogens also affect pathological vascular processes such as angiogenesis, inflammation, tissue damage caused by ROS, and can inhibit atherosclerosis development (Gencel et al., 2012).

## CONCLUSION

This study provides compelling evidence regarding the effects of Phaleria macrocarpa flavonoid extract on VEGF expression in the endometrium and myometrium of menopausal mice models. The research demonstrated that administration of Phaleria macrocarpa flavonoid extract at doses of 7.5 mg/mouse/day, 11.25 mg/mouse/day, and 15 mg/mouse/day significantly decreased VEGF expression levels in both tissues. These findings are supported by significant differences observed in VEGF expression between treatment groups and controls, as indicated by One-Way ANOVA and Tukey's Honestly Significant Difference (HSD) test results. Moreover, Pearson correlation analysis revealed a strong inverse relationship between the doses of Phaleria macrocarpa flavonoid extract and VEGF expression in the endometrium (r = 0.600) and myometrium (r = 0.637). This correlation suggests that higher doses of the extract lead to decreased VEGF expression, highlighting the potential of Phaleria macrocarpa flavonoids to modulate angiogenic processes in menopausal uterine tissues. These findings contribute to the understanding of phytoestrogen-based therapies for menopausal health management, emphasizing the role of natural compounds in alleviating vascular and hormonal disruptions associated with menopause. Future research should explore additional mechanisms underlying these effects and evaluate long-term impacts to further validate the therapeutic potential of Phaleria macrocarpa flavonoid extract in clinical settings.

## CONFLICT OF INTEREST

The author disclosed that there is no significant issues were found in this study.

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