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The Impact of *Phaleria macrocarpa* Fruit Flavonoid Extract on Endometrial Thickness in Mice Menopausal Model

***Dyah Ayu Septika Wijaya[1], Recavery Dwi Wulandari[1], Sutrisno[2], Yahya Irwanto[2], Kenty Wantri Anita[4]**

[1]Master Program of Midwifery, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia, [2]Departement of Obstetrics and Gynaecology, Faculty of Medicine, Universitas Brawijaya, Dr. Saiful Anwar General Hospital, Malang, East Java, Indonesia, [4]Department of Clinical Pathology, Faculty of Medicine, Universitas Brawijaya, Malang, East Java, Indonesia**.** *Email: a.dyahwijaya@gmail.com
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Abstract: This study aims to determine how the effect of flavonoid phaleria macrocarpa fruit extract on the endometrial thickness of Mus musculus which is used as an experimental animal with a menopause model. This study employed a randomized post-test-only control group design on 32 female mice. Ovariectomy simulated menopause, followed by treatment with flavonoid from *Phaleria macrocarpa* fruit extract. Post-observariectomized, follicle-stimulating hormone levels indicated menopausal status. Hematoxylin-Eosin methods assessed endometrial thickness. The result indicated reduced endometrial thinning in the positive control group (K+). Conversely, the treatment group (P1-P4) exhibited increased endometrial thickness post-flavonoid extract administration. Ovariectomized-induced estrogen decline resulted in reduced endometrial thinning, mimicking menopausal conditions. Statistically, flavonoid extract administration increased endometrial thickness in ovariectomized-induced menopausal mice, with optimal effect observed at 7.5 mg/mice/day for 14 days. The preferred intervention dose for altering endometrial thickness in menopausal conditions was identified as P1 (3.75 mg/mice/day). In conclusion, a flavonoid from *Phaleria macrocarpa* fruit extract mitigates endometrial thinning in the menopausal mice model. For implication in menopausal women, clinical trials need to be conducted to evaluate the dosage of these flavonoids for their potential as a natural SERM (Selective Estrogen Receptor Modulator) before clinical application in managing endometrial health. Additionally, research on molecular effects particularly regarding ER (Estrogen Receptors) in the endometrial organ, is needed to confirm their phytoestrogenic effect.
Keywords: Endometrial thickness; flavonoid; *Phaleria macrocarpa*.

INTRODUCTION

Menopause, derived from the Greek words “men” (month) and “pause” (stop), signifies the termination of menstrual cycles. It occurs when menstruation is absent for 12 consecutive months due to a decline in estrogen levels produced by the ovaries, reduced follicular activity, and the ovaries’ diminished responsiveness to hormonal signals (Romadhona et al., 2022). Typically occurring between the ages of 45-55 and premature menopause may occur before 40 due to autoimmune disorders, chromosomal abnormalities, or medical intervention like hysterectomy, hormonal contraceptives, or medications(WHO, 2022). By 2030, the global post-menopausal female population is projected to reach 1.2 billion (The World Bank, 2022). In Indonesia, life expectancy increased from 70.1 years (2010-2015) to 72.2 years (2030-

Corresponding Author: Dyah Ayu Septika Wijaya
Master Program of Midwifery, Faculty of Medicine, Universitas Brawijaya,
Jalan Veteran Malang 65145, East Java, Indonesia
Email: a.dyahwijaya@gmail.com

2035). Longer lifespans increase the likelihood of reaching menopausal age. Indonesia's menopausal women population in 2021 increase from 0.12% to 12.64% (Badan Pusat Statistik, 2013).

In the physiology of the menstrual cycle, the pituitary gland produces follicle-stimulating hormone (FSH) and luteinizing hormone (LH), while the ovaries generate estrogen and progesterone. Estrogen prompts follicles to release eggs, increased estrogen and progesterone, thickening the endometrial lining. However, declining ovarian productivity reduces follicle production, resulting in decreased estrogen and progesterone, ovarian follicle atresia, and menstruation cessation. Menopause is characterized by FSH levels exceeding 35 mIU/ml and estradiol levels below 30 pg/ml. The hypothalamus, detecting estrogen deficiency, releases GnRH to the anterior pituitary, triggering FSH and LH release. Despite elevated FSH and LH, aging ovaries remain unresponsive, causing irregular menstruation. Elevated FSH and LH levels persist, lacking negative feedback mechanisms. Estrogen production ceases as remaining primordial follicles become atretic. Histologically, reduced ER responses lead to no endometrial shedding. Post-menopause, estradiol transforms into estrone, ultimately becoming estrogen. Estrone is produced by the ovaries and adipose tissue, and stored as estrone sulfate (Brenda & Janke, 2022; Romadhona et al., 2022).

Hypoestrogenic conditions during menopause induce physical, emotional, mental, and social changes and long-term risks. Physical changes like sagging breasts, thinner fallopian tube folds, vanishing vaginal rugae, increased vaginal pH, and altered secretions, pose potential health risks, particularly for reproductive organs such as the uterus. These changes lead to fluid loss and alterations in interstitial connective tissue, leading to vulnerable thinning epithelial cells. Concerning the endometrium, hypoestrogenic induces atrophy, hyperplasia, and an increased risk of endometrial cancer. These effects stem from changes in interstitial tissue with thin and easily injured epithelial cells. These alterations are a consequence of decreased estrogen levels, with ER no longer responding to stimulation, causing a lack of ER expressing proliferation in the endometrial wall (Romadhona et al., 2022; WHO, 2022).

Magnetic resonance (MR) examinations prove the condition of the uterus in menopause, the endometrium thickens during the secretory phase, the myometrial signal increases due to water content and vascular flow, and low-intensity uterine contractions form the uterine contour. Post-menopausal phase reveals endometrial atrophy, with a loss of border zones (Nori, 2021). Transvaginal ultrasound (TVUS) imaging during menopause depicts a thin or atrophic endometrium. Post-menopause, endometrial walls may exhibit proliferative activities, including atrophy, simple hyperplasia, complex hyperplasia, atypical hyperplasia, or endometrial cancer. These observations are also evident in histological examinations using hematoxylin-eosin (H.E) staining methods (Otify et al., 2015; Swain & Kulkarni, 2021).

Pharmacological and hormonal interventions are commonly administered to address symptoms and issues faced by menopausal women. However, based on studies, some of these therapies can cause direct and long-term side effects, as well as certain contraindications, such as : (1) Menopausal Hormone Therapy (MHT) addresses symptoms and prevents endometrial hyperplasia or cancer (Deligdisch-Schor & Mareş Miceli, 2020). Contraindications involve conditions like breast cancer, cervical cancer, severe liver dysfunction, abnormal vaginal bleeding, coronary heart disease, meningioma, myocardial infarction, and prolonged use raises the cancer risk (Ariyanti & Aprilia, 2016); (2) Selective Serotonin Reuptake Inhibitors (SSRI) and Serotonin-Norepinephrine Reuptake Inhibitors (SNRI) for vasomotor symptoms (Markovac & Marcus, 2020) with notable side effects include nausea and constipation.

So it should be noted for use on hypertensive patients due to potential blood pressure elevation(Stubbs et al., 2017); (3) SERM, acting as an estrogen antagonist in breast tissue but exhibiting agonistic effects on the uterus (Eyster, 2022; Fait, 2019; Markovac & Marcus, 2020).

Therefore, due to the side effects, contraindications, and agonist-antagonist properties in the use of pharmacological and hormonal therapies, some researchers implement phytochemical treatments. Phytochemical compounds found in plants that offer numerous benefits to humans. Non-steroidal estrogen from plant products with estrogenic activity is known as phytoestrogen(Lecomte et al., 2017). Flavonoids serve as a primary group of phytoestrogens with agonistic properties(Kiyama, 2023). Their estrogenic effects are mediated by two distinct ER (ERα and ERβ), which can regulate various biological activities such as cell growth and differentiation (Lecomte et al., 2017). Flavonoids influence cell proliferation through various mechanisms, including interaction with hormonal signaling pathways and binding to ER (Vachetta et al., 2022). Previous research on flavonoids from the *Cuscuta chinensis* plant in female mice exposed to bisphenol (BPA) showed the ability of flavonoids to improve reproductive structure and function and regulate hormonal balance. Flavonoids increase the expression of hormones (E2, P4, FSH, LH) and reproductive receptors (ERα and ERβ) as well as reduce apoptosis in the ovaries(Han et al., 2020). Meanwhile, according to another study, flavonoids in *Medicago saliva have* the potential to enhance the endometrial epithelium of the uterus in OVX rats (Jdidi et al., 2021).

Phaleria macrocarpa plant originates from Indonesia, particularly from the region of Papua. This plant has been recognized for its diverse medicinal properties capable of treating various ailments with flavonoid content (Sutrisno et al., 2023). Ethanol extraction reveals compounds such as phalerin, mahkoside A, dodecanoic acid, palmitic acid, des-acetyl flavicordin-A, flavicordin-D, flavicordin-A glucoside, ethyl stearate, and lignin sucrose (Mamatha et al., 2020). In another study, 96% ethanol solvent reveals tannin, flavonoid, alkaloid, glycoside, and saponin. Flavonoid content in ethanol extracts of *Phaleria macrocarpa* fruit varies between 3.41% and 12% (Mahayuni & Wirasuta, 2023). Some specific flavonoids found include kaempferol, myricetin, naringin, and rutin (Easmin et al., 2015; Maharani, 2023). The entire part of *Phaleria macrocarpa* has been traditionally used in herbal medicine. This includes addressing symptoms, issues, and risks that may occur in women during and after menopause, such as insulin resistance (Ali et al., 2012), lipid metabolism changes, fat and cholesterol redistribution, reproductive organ cancers including breast cancer (Ahmad et al., 2023), muscle, and joint mass reduction (Irawan et al., 2022), decreased bone density (Mustaqim et al., 2021), and cardiovascular disease risks (Rumahorbo et al., 2023).

In the body, flavonoid as a phytoestrogen binds to ER in the cytoplasm when endogenous estrogen is deficient. This binding results in an active receptor complex that combines with DNA and undergoes transcription. The expression of receptor protein depends on the estrogen target organ and its receptor. ER α and β are abundant in reproductive organs. In the uterus, flavonoids binding to ER can influence the alveolar cavity and globular particle system growth in the uterine glands, affecting the endometrial wall's proliferation, increasing vaginal elasticity, and other menopausal symptoms (Ariyanti & Apriliana, 2016).

Research on menopause has also been conducted on ovariectomized (OVX) rats. Rats were treated with electroacupuncture (EA), and then examinations were performed on the uterine morphology and ER expression. Observations were carried

out on 30 rats (10 control group, 10 OVX, 10 OVX + EA) for 3 days. Examinations were conducted using H.E, qRT-PCR, and western blot, revealing that OVX + EA rats showed an increase in uterine weight, thickening of the endometrium, elevated ER protein, and reduced atrophy (Ma et al., 2017).

There has been research on menopause in ovariectomized (OVX) rats with electroacupuncture (EA) treatment, but there is still limited research on treatment using phytoestrogens. So, this study aims to analyze the effect of phytochemical content, especially flavonoid extract from *Phaleria macrocarpa* fruit, on the endometrial thickness of mice in the OVX menopause model. This could serve as an alternative prevention for women facing short-term and long-term effects of menopause, including symptoms and the risk of complications, particularly in the reproductive organs. The hypothesis posited that the flavonoid extract from *Phaleria macrocarpa* fruit could increase endometrial thickness in the menopause mice.

MATERIALS AND METHODS

Research Design

This study used a true experimental research design utilizing a randomized post-test-only control group approach. The research has undergone ethical review and approval by the Research Ethics Commission of Brawijaya University, with the assigned protocol No. 27/EC/KEPK-S2/01/2024.

Animal

The author utilized female mice (*Mus musculus*) aged 6-8 weeks, weighing between 20-30 grams, and ensuring they were in a healthy condition. A total of 32 experimental animals were employed, divided into two groups: Negative control (K-) (n=6) and the OVX group (n=26). OVX was performed after a 7-day acclimatization period in the positive control group using a combination of 80 mg/kg ketamine and 10 mg/kg xylazine for anesthesia. After 28 days, the menopausal status was confirmed through FSH examination in one mouse from each group. Subsequently, the OVX group was grouped into five categories: K+ (OVX) n=5; P1 (OVX + 3.75 mg/mice/day) n = 5; P2 (OVX + 7.5 mg/mice/day) n = 5; P3 (OVX + 11.25 mg/mice/day) n = 5; and P4 (OVX + 15 mg/mice/day) n = 5. The flavonoid extract doses for each group were administered orally for 14 days using a gastric sonde, following established research protocols (Maharani et al., 2021).

Preparation of Flavonoid Extract

Phaleria macrocarpa fruits were obtained from Batu City, East Java, and processed at the Materia Medika Batu laboratory. The location and extraction process was consistent with those of the preceding study (Maharani et al., 2021). The portion of the fruit utilized in this study comprises the mesocarp and pericarp distinguished by a maroon hue on the fruit's skin, with the seeds removed. The flavonoid extract from the mesocarp and pericarp of *Phaleria macrocarpa* fruit was obtained through an herbal extraction technique of maceration. Dry powdered raw material weighing 2500 grams was finely ground and soaked in 30L of 90% ethanol solvent. The mixture was stirred for 30 minutes, left to stand for 5 days, filtered using a buncher funnel to obtain the macerate, and evaporated at 60ºC. Subsequently, fractionation was conducted using n-hexane and n-butanol to isolate the flavonoid compounds. The process involved dissolving the ethanol extract in n-hexane, homogenizing, including separation with n-hexane, and repeating three times. After obtaining the precipitate, n-hexane is evaporated at approximately 45ºC. Flavonoid compounds were then isolated using n-butanol partitioning, repeated three times. Then supernatant is

evaporated at around 60ºC, yielding a concentrated flavonoid paste (Maharani et al., 2021).

Endometrial Thickness Analysis

The organ retrieval procedure was conducted, utilizing minor surgical tools and peritoneal anesthesia with 0.2 ml of 1% ketamine. The surgical steps include making a ventral transverse incision, collecting the uterus, and immersing in a 10% formalin fixative buffer. Histopathological preparations include longitudinal cutting on the uterus horn, subsequent cutting, deparaffinization, Hematoxylin-Eosin (H.E) staining, and mounting using Entellan. Endometrial thickness measurements were performed on 30 histological preparations, with data collected from 10 quadrants using the Olympus XC10 dot slide microscope with a magnification of 400x.

Statistical Analysis

Data are presented as mean ± SD. ANOVA test was conducted to analyze the differences in means between treatment groups. Post Hoc with Tukey (Table 2.) was used for specific tests to handle multiple comparisons and identify dosage groups that significantly differ from each other, while Pearson was utilized to test the strength between flavonoid doses using the SPSS 26.0 software as a statistical program. If $p>0.05$, it is concluded that there is a significant difference among the six groups.

RESULTS AND DISCUSSION

Based on the result, the concentration of FSH was measured in two experimental groups, the K- group was identified at 1.30 ± 0.40, and the OVX group at 6.19 ± 0.19 (Table 1). The significant difference is indicated by an approximately ~4,76 fold. This suggests that the non-OVX and OVX groups have different FSH levels, implying that mice with high FSH concentrations are in a menopausal condition. This aligns with the theory stating that the expected FSH increase in the treatment groups is approximately ~40-fold when compared to the control group (Yousefzadeh et al., 2020). The actual percentage difference is 376.15%, exceeding the theoretical increase of 222.2% in FSH levels for bilateral OVX mice after 4 weeks (Rodríguez-Landa, 2022).

The percentage calculations from each viewpoint were subsequently analyzed using a statistical test. The result above (Table 2 and Figure 2), indicates that the highest average thickness of endometrium was found in the P2 group (OVX + 7.5 mg/mice/day flavonoid for 14 days) at 162.064 ± 40.203008, followed by the second-highest average thickness in the P1 group, at 150.3408 ± 27.780794. Meanwhile, the lowest average thickness of endometrium was observed in the K+ group, at 98.5886 ± 10.974973. Further, One-Way ANOVA analysis was conducted to examine whether there were significant differences between the treatment groups. Before this, normality and homogeneity tests were performed to ensure that the data met the assumption of the analysis. The normality test (Table 2) using Shapiro-Wilk indicated that in all treatments, $p > 0.05$, suggesting that the endometrial thickness data tended to be normally distributed in each treatment group. Subsequently, the homogeneity test was conducted using Lavene's test, with results showing $p > 0.05$, indicating that variance homogeneity was met among the treatment groups. With normal and homogeneity assumptions met, further analysis was conducted using ANOVA, which showed a significance value of $p < 0.05$, indicating significant differences in endometrial thickness between the treatment groups.

Table 1. The Concentration of FSH Levels by Non-OVX and OVX

Group	Replication		Average (IU/L)	SD
	1	2		
K-	1.01	1.58	1.30	0.40
OVX	6.32	6.05	6.19	0.19

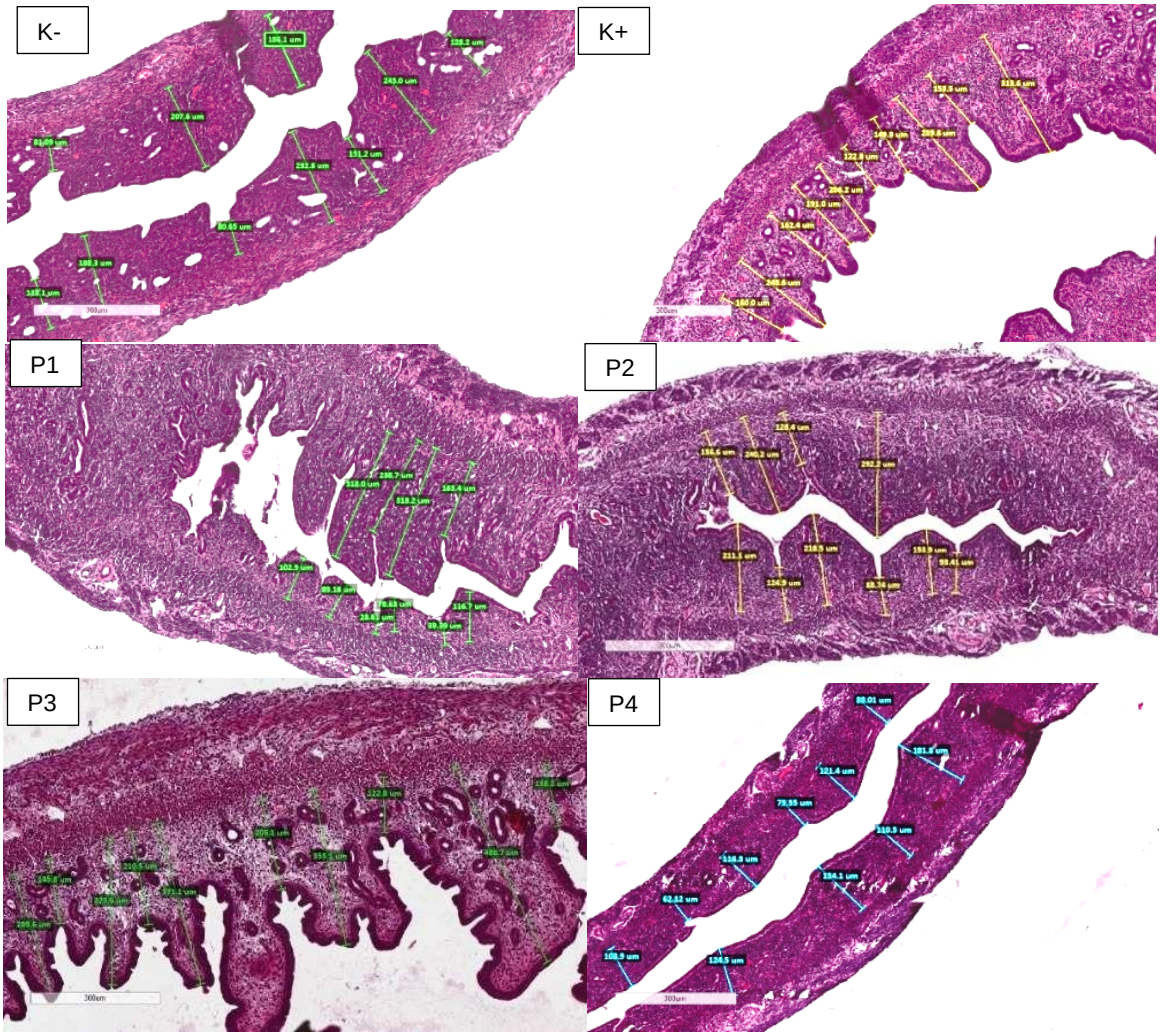

Figure 1. Microscopic images of the mice endometrium were stained with H.E. and scanned using Leica Aperio CS2. Observations were conducted using Image Scope software at a magnification of 80x at 10 points, comprising 5 highest points and 5 lowest points. Endometrial thickness was measured and indicated by lines.

The Significant difference between K+ and P2 groups indicates that the administration of flavonoid extract significantly increases the thickness of the endometrium affected by OVX. This finding is consistent with the role of flavonoids as phytoestrogens that can mimic the effects of endogenous estrogen, including enhancing the growth of endometrial tissue (Ariyanti & Apriliana, 2016). The administration of flavonoids as phytoestrogens can increase the activity of endometrial cell proliferation. Estrogen stimulates the endometrial cell to replicate and grow, resulting in thicker endometrium (Bartiromo et al., 2021).

Table 2. Endometrial Thickness Average Results Each Treatment Group

Treatment Group	Endometrial Thickness		
	Mean (%)	SD	Post Hoc Notation[1] (symbol of test area)
K-	182.85500	40.977279	b
K+	98.58860	10.974973	a
P1	150.34080	27.780794	ab
P2	162.06400	40.203008	b
P3	145.38720	35.328551	ab
P4	144.02300	39.206233	ab
Sig. of normality	= .297		
Sig. of homogeneity	= .399		

[1]Post hoc notation is obtained from the Tukey test. “a”, “b”, and ”ab” refer to the comparation results between different groups in statistical analysis. This the comparation results between different groups in the statistical analysis. This indicates significant differences between specific groups: “a” indicates that the mean of the first group is significantly different from the mean of the second group; “b” indicates thet the mean of the second group is significantly different from the mean of the first group; “ab” indicates that there is no significant difference between the means of the first and second group. In other wirds, “a”, “b”, and “ab” indicates no significant difference between two group.

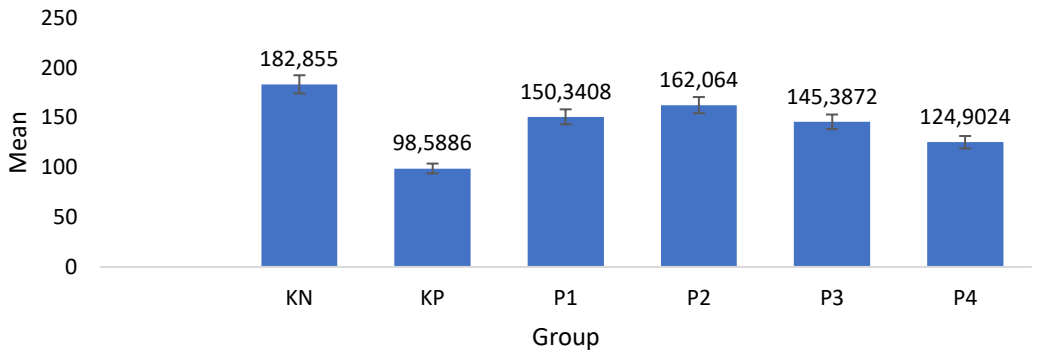

Figure 2. Average Endometrial Thickness Values Bar Graph

Table 3. ANOVA Test on Endometrial Thickness Result

Variable	Sig.	Conclusion
Endometrial Thickness	0.005	Significant

Based on these results (Table 3) a significant value of p< 0.05 was obtained, indicating that different treatments had a significant effect on endometrial thickness in mice. Post-hoc analysis was performed using Tukey’s Honestly Significant Difference (HSD) method to compare and determine differences between all pairs of groups. The results of this test are displayed in the notation form in the statistical analysis table (Tab.2). Subsequently, statistical analysis to assess the relationship between

flavonoids and endometrial thickness was conducted using Pearson correlation analysis (Tab 4.). The results showed that the significance value of $p < 0.05$ for the endometrial thickness parameter group indicated a significant relationship between flavonoid dose and endometrial thickness. It can be concluded that the flavonoid extract of *Phaleria macrocarpa* fruit has a significant effect on the endometrial thickness parameter in the observed samples.

Table 4. The Result of Pearson Correlation Analysis of Flavonoid Extract from *Phaleria macrocarpa* Fruit with Endometrial Thickness

Variable	Correlation Coefficient	Sig.	Conclusion
Endometrial Thickness	0.685	0.005	Significant

Additionally, simple linear regression analysis was conducted to determine the effect of variables on each other. In this data processing, analysis was performed on the dose of flavonoid extract of *Phaleria macrocarpa* fruit on endometrial thickness. Tab.5 shows that the dose is a variable X with a significance value of $p< 0.05$ and a R_{square} of 0.470, indicating that the flavonoid extract dose of *Phaleria macrocarpa* fruit has a 47% influence on endometrial thickness.

Table 5. Regression Summaries of Flavonoid Dose on Endometrial Thickness

Variable	B	t_{value}	Sig.	Conclusion
Constant	105.260			
Dosage	8.463	3.394	.005	Significant
R^2	= .470			

The Pearson correlation analysis aligns with the results of linear regression, which confirms that Flavonoid *Phaleria macrocarpa* fruit extract has a positive influence on endometrial OVX mice as a menopausal model. The increase in thickness remains at normal levels compared to the K- groups as the control group. Based on the findings, *Phaleria macrocarpa* flavonoid has the potential as a natural hormone replacement intervention or therapy to address endometrial thinning and associated risks during menopause. In menopause conditions, endometrial atrophy leading to abnormal and irregular bleeding and pelvic pain can be intervened using the dosage in P1 (3.75 mg/mice/day) as the ideal and safe dosage selected by researchers. With an increase in thickness not significantly different from P2 (7.2 mg/mice/day), this dosage may be suitable for women experiencing early menopause under the age of 40 who have never been pregnant to aid fertility.

In the context of selecting the ideal dosage, the dosage or supplements is a crucial measure in achieving the desired effects on health. When the dosage administered is too low, it may result in therapy not achieving the expected effect, while dosages that are too high have the potential to cause harmful side effects or risks to bodily organs. The ideal dosage for intervention in conditions involving changes in endometrial thickness in menopausal women can be determined based on research findings that identify effective doses in eliciting the desired treatment effects (Tyson et al., 2020). The selection of dosage P1 is made based on several considerations related to effectiveness in achieving therapy goals and the body's tolerance to the dosage to be administered.

Based on the bar graph (Figure 2) presented in the previous chapter, it is also shown that the effect of flavonoid administration exhibits similar trends in endometrial

thickness. However, as the dosage increases, the endometrial thickness. This demonstrates the concept of hormesis, which suggests that although high doses of flavonoids may be administered, they can lead to a decrease in effectiveness due to factors such as low bioavailability, interaction with other drugs, and even toxic effects. Conversely, when administered at low doses, they are more effective as they can avoid unwanted side effects and produce positive effects or protection. Additionally, considering the low bioavailability of flavonoids, low doses may already be sufficient to achieve the desired therapeutic effect (Calabrese et al., 2010).

This study only focuses on the use of flavonoid extract from Phaleria macrocarpa fruit without showing the specific flavonoid subclass in expressing endometrial thickness. Future research should focus on the specific flavonoid subclasses of Phaleria macrocarpa fruit extract that affect estrogen or progesterone receptors and binding affinity at the molecular level in menopausal animal endometrial tissue models.

CONCLUSION

The conclusion in this study shows that flavonoid extract from phaleria macrocarpa fruit has an effect on the thickness of the endometrium of the menopausal Mus musculus model in OVX with a dose of P1 (3.75 mg/mice/day), with the optimal effect observed at 7.5 mg/mice/day for 14 days. The preferred intervention dose for altering endometrial thickness in menopausal conditions was identified as P1 (3.75 mg/mice/day), effectively addressing endometrial atrophy and its associated effects, as well as reducing the risk of infection in the inner layer of the uterus, up to endometrial cancer to the menopausal mice model. Based on theoretical review and research findings, it is revealed that flavonoids are secondary metabolites in the form of phytoestrogens. In this study, flavonoids obtained from the fruit of *Phaleria macrocarpa* were tested on a menopausal mice model. However, to confirm its molecular effects as a phytoestrogen agent, further research is needed on ER in the endometrial organ. Moreover, the potential activity of these flavonoids requires clinical trials for application in clinical or medical practice as a natural SERM before implementation in human subjects as suggesting therapeutic in managing menopausal symptoms, reducing short-term risk such as abnormal and irregular bleeding, pelvic pain due to endometrial atrophy, and long-term risks such as infections in the inner layer of the uterus, up to endometrial cancer.

CONFLICT OF INTEREST

The author declares that no significant issues were found in this research.

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