The Effect Flavonoids Phaleria macrocarpa Fruit Extract on Thickness of Trabeculae, Cortex Ratio Femoral Bone and Aortic Intima-Media in Mice Menopause Model

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Abstract

A deficiency of the hormone estrogen at menopause can lead to an increased rate of the destruction of the bone tissue that leads to bone loss, which can lead to osteoporosis and impaired fat metabolism, which increases the risk of atherosclerosis. Phytoestrogens from flavonoid extract P. Macrocarpa, having effects similar to endogenous estrogens themselves, prevent osteoporosis and atherosclerosis in menopausal women. The purpose of this research is to assess the influence of flavonoids from P. Macrocarpa fruit extract on trabeculae cortex thickness, ratio of femoral bone, and aortic IMT (A-IMT) in a menopausal mouse model. The study was conducted in a true experimental-posttest-only control group design. Using 32 mice; namely KN (normal mice with no treatment), KP (OVX with no treatment), P1(OVX and given flavonoid 3.75 mg/mice/day), P2 (OVX and given flavonoid 7.5 mg/mice/day), P3(OVX and given flavonoid 11.25 mg/mice/day), P4 (OVX and given flavonoid 15 mg/mice/day), the treatment given within 14 days. Then the thickness of the trabeculae, cortex, and intima-media aorta with Hematoxylin-Eosin (HE) staining. In the trabeculae, cortex thickness ratio obtained KN results meaningfully dissimilar to the KP group and the P3 and P4 groups were meaningfully dissimilar from the KP. The A-BMI in KP is meaningfully dissimilar to P1, P2, P3 and P4. The conclusion of the study is flavonoid fruit extract P. Macrocarpa can increase the thickness ratio of trabeculae, and cortex femoral bone of mice menopausal model in groups P3, P4 and can decrease A-IMT starting in groups P1 to P4.

Keywords: Flavonoid, Phaleria macrocarpa, Menopause, Osteoporosis, Atherosclerosis.

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1. INTRODUCTION

Although literally, menopause means the cessation of menstruation, in a broader sense, it means the menstrual cycle terminating permanently as a result of ovarian follicular activity declining and estrogen levels falling (Silva et al., 2021; WHO, 2022). The regularity and length of a woman’s menstrual cycle vary widely throughout her reproductive life, although the age of natural menopause commonly occurs in women globally aged 45 to 55 (WHO, 2022). 4.3 million women in Indonesia aged 45 to 55 years. In 2017, Indonesia’s population reached 261.89 million, consisting of 130.31 million women aged 45 to 55 years, and it is estimated that there are 15.8 million women who are menopausal in 2020. This indicates that there will be more menopausal women in Indonesia by 2020 (BPS, Bappenas dan UNFPA Indonesia, 2008). Menopause is referred to as failure and the beginning of disease (Macpherson & Quinton, 2022).

Osteoporosis is one of the most frequent disorders among postmenopausal women because a lack of the hormone estrogen after menopause can accelerate bone resorption and disrupt the bone-rebuilding process. 40% of Indonesian women are susceptible to osteoporosis (Juwita & Fatma, 2021). Osteoporosis is a condition characterized by reduced bone density and deterioration of the microscopic structure of bone tissue, resulting in increased vulnerability to fractures and an increased risk of fractures (Liu, 2020; NIH, 2022). Osteoporosis is primarily influenced by aging and the reduction in steroid hormone levels (Geng et al., 2019).

Cardio Vascular disease (CVD) caused by atherosclerosis, also known as Atherosclerotic Cardiovascular Disease (ASCVD), is more commonly found in Canadian women with an average age of 69 years (Hopper et al., 2021; Newson, 2018). Estrogen levels can also be associated with impaired fat metabolism that has a risk of lipid peroxidation, leading to atherosclerosis (Newson, 2018). Hypercholesterolemia can be caused by a decrease in estrogen that occurs in menopausal women. Low Density Lipoprotein (LDL) levels increase in this condition. Monocytes enter the tunica intima and become macrophages due to increased LDL in the subendothelium (Javidifar et al., 2021; Pratiwi & Damayanty, 2020). To produce atheromatase plaques, scavengers on macrophages can identify Ox-LDL and engulf it into foam cells (Khatana et al., 2020; Sargowo, 2015).

Hormone replacement therapy, (HRT), is given to individuals experiencing hormonal disorders, one of which is menopausal women receiving estrogen hormone therapy (Nayak et al., 2022). Several studies have shown that HRT lowers fracture risk and may be used to prevent or treat osteoporosis in postmenopausal women (de Villiers, 2023; Women Health Concern, 2021). In addition, it is known that estrogen hormone therapy can also reduce atherosclerosis and fat accumulation in menopausal women (Khoudary et al., 2020). However, long-term side effects such as breast cancer, uterine cancer, impaired liver function, and vaginal bleeding are still a debate about the use of HRT (Yousefzadeh et al., 2020). In addition, menopausal women are afraid to accept prescriptions and undergo HRT therapy (Macpherson & Quinton, 2022).

Therefore, additional preventive alternatives are needed, especially natural compounds that have minimal side effects, namely phytoestrogens (Bacciottini et al., 2007). Flavonoids of Phaleria macrocarpa fruit, known as mahkota dewa, are widely found in nature, especially in Indonesia, and are one of the phytoestrogens (Ahmad et al., 2023). The bioactive compounds of the Phaleria macrocarpa plant include flavonoids (Stephenus et al., 2023). Flavonoids found in Phaleria macrocarpa fruit function as antimicrobial, antibacterial, antifungal, antiallergic, antioxidant, and vasodilator (Fitriana et al., 2023). The highest relative levels of flavonoids were found in the 70% ethanol extract of the flesh of the Phaleria macrocarpa fruit, which reached 45.734 g/mg (Maharani et al., 2021). Phytoestrogens in plants have the ability to bind to estrogen receptors (ER) to mimic or modulate endogenous estrogen activity. This
endogenous estrogen is 17β-estradiol, mainly by binding to the ER. Aside from impacting estrogen receptors, phytoestrogens can also function as antioxidants (Forslund & Andersson, 2017; Hasanah et al., 2020; Kuhnle et al., 2009).

The novelty of this research is to see the effect of giving *Phaleria macrocarpa* fruit extract where flavonoids isolated specifically can be one of the active substances for phytoestrogens. Other studies on the effects of flavonoids of *Phaleria macrocarpa* fruit extract have given good results such as research on endometriosis, diabetes, and other diseases, but until now there have been no studies related to menopause. Therefore, this research aims to ascertain how flavonoids from *Phaleria macrocarpa* fruit extract affect the ratio of trabecula thickness and femoral bone cortex and can reduce the thickness of the intima-media aorta of mice in menopausal models. Through this research, it is hoped that flavonoids of *Phaleria macrocarpa* fruit extract can be an alternative preventive innovation in reducing the incidence of osteoporosis and atherosclerosis, especially in women with menopause.

2. RESEARCH METHOD

This study uses a true experiment research design conducted on female mice (Mus musculus). Randomized Post-Test Only Control Group Design was used as the research design. The research also used in vivo methods to see how flavonoids of *Phaleria macrocarpa* fruit extract impacted menopausal models. A place for ovariectomy treatment in mice, where the treatment is given and the tissue collection process is carried out at the Embryology Laboratory, Faculty of Veterinary Medicine, Airlangga University, Surabaya. Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Brawijaya Malang as a place for making and observing research preparations. In this study, 32 mice (Mus musculus) were used as experimental animals; 2 mice were used as samples to test FSH levels to ensure that the mice were in menopause and the allocation of 30 mice was partitioned into six groups, with each group including five mice.

The negative control group (KN) comprises subjects receiving no treatment, while the positive control group (KP) consists of subjects undergoing ovariectomy treatment, where before the division of treatment groups 1 to 4, 2 samples of mice were taken to ensure that mice were in a state of menopause characterized by increased FSH results on the 28th day after ovariectomy. The experimental groups designated as P1, P2, P3, and P4 were groups that carried out ovariectomy treatment and were given flavonoids *P.macaropa* fruit extract at doses of 3.75 mg/mice/day, 7.5 mg/mice/day, 11.25 mg/mice/day, and 15 mg/mice/day for 14 days. The dosage in this study is based on previous research by Maharani et al (2021) which investigated the impact of flavonoids derived from *P.macaropa* fruit extract on endometriosis mice. The study stated that flavonoids of god's crown fruit extract were obtained through herbal extraction techniques using 96% ethanol solvent because 96% ethanol is semi-polar resulting in higher levels can be caused by flavonoids containing more is nonpolar so that flavonoid levels are obtained (Maharani & Sutrisno, 2021; Pujiastuti & El’Zeba, 2021).

Making flavonoids Phaleria macrocarpa fruit extract is a ripe fruit washed, the seeds are removed, and the fruit is dried not in the sun to be free from water protection. Simplisia powder is made by blending dried Phaleria macrocarpa. Simplisia powder is soaked in 96% ethanol for 30 minutes, stirred well, and allowed to stand for 5 days until settled. Using a funnel of bunches, strain the liquid. To obtain flavonoid-rich extracts, ethanol extracts are partitioned/liquid-liquid fractionated using polar and nonpolar solvents, namely n-hexane and n-butanol.

After being treated for 14 days. Then surgery was performed and examination of thickness of trabeculae, cortex ratio femoral bone, and aortic intima-media in the menopausal mice model with Hematoxylin-Eosin (HE) staining. In this study, the ratio of the thickness of trabeculae and cortex, namely the thickness of the trabeculae in the metaphysis area and the thickness of the cortex in the diapase area, was observed by making a thickness of 3-4μm, using
1 field of view with 10 measures and calculating the average of each preparation. Measurement of intima-media thickness seen from 4 viewing lights (directions at 3, 6, 9, and 12 o'clock) descending thoracic aorta, organs that have been stained and then scanned using Aperio CS2 Leica and calculations using the ImageScopex64 application.

The thickness of trabeculae, cortex ratio, femoral bone, and aortic intima-media in the menopausal mice model were statistically analyzed using IBM SPSS Statistics 27.0 for Windows. The tests employed in this work encompass data normality assessments utilizing the Shapiro-Wilk test, data homogeneity evaluations employing the Levene Test method, One Way ANOVA Test, and Post Hoc Test.

The procedures employed in this inquiry strictly complied with the applicable norms and regulations and obtained clearance from the Health Research Ethics Committee of the Faculty of Medicine, University of Brawijaya Malang, Indonesia, under ethics code numbers: 108/EC/KEPK-S2/05/2024 and 26/EC/KEPK-S2/01/2024.

3. RESULTS AND DISCUSSION
a. Ratio of Trabeculae and Cortex Thickness in Femoral Bone of Mice Menopause Model

The results of staining the thickness of trabeculae and femoral bone cortex of mice menopausal models carried out Hematoxylin-Eosin (HE) staining and observations were made using Aperio ImageScopex64 software with a magnification of 50-100X with the treatment of giving flavonoids Phaleria macrocarpa fruit extract with 4 different doses, the following results were obtained:

Figure 1. Histopathology of the femoral bone of mice. Histopathological picture of trabeculae bone in the area of the metaphysis (A, B), cortex bone in the area of the diaphysis (C, D). Image with 50-100x magnification with HE coloring
Table 1. One-way ANOVA Test Results of Trabeculae and Cortex Thickness Ratio in Femoral Bone

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean ± SD (Thickness (µm))</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN</td>
<td>0.426 ± 0.157</td>
<td></td>
</tr>
<tr>
<td>KP</td>
<td>0.156 ± 0.010</td>
<td></td>
</tr>
<tr>
<td>P1</td>
<td>0.311 ± 0.054</td>
<td>0.020</td>
</tr>
<tr>
<td>P2</td>
<td>0.327 ± 0.174</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>0.399 ± 0.087</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>0.408 ± 0.161</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 illustrates that the positive control group (KP), which is a group that was only given ovariectomy treatment to make mice menopause model, the average ratio of trabeculae and cortex thickness decreased to 0.156 µm compared to the negative control group (KN) or normal mice, which is 0.426 µm, where the significance value p = 0.022 (p<0.05), this indicates that there is a significant difference in each treatment group (P1-P4) after administering flavonoids of Phaleria macrocarpa fruit extract with different doses for 14 days.

This study has also proven that using 4 doses of flavonoids in Phaleria macrocarpa fruit extract can increase the ratio of trabeculae and cortex thickness. There was an increase in the ratio of trabeculae and cortex thickness in the intervention group 1 (P1) treated with flavonoid Phaleria macrocarpa fruit extract at a dose of 3.75 mg/mice/day and intervention group 2 (P2) with a dose of 7.5 mg/mice/day with an average result of 0.311 µm and 0.327 µm, the results increased when contrasted with the average of the KP group yield of 0.156 µm. In intervention group 3 (P3) and intervention group (P4), there was a statistically meaningful increase compared to the KP group. The mean values for P3 and P4 were 0.399 µm and 0.408 µm, respectively.

Table 2. Tukey HSD Test Results Against Trabeculae and Cortex Thickness Ratio Data

<table>
<thead>
<tr>
<th>p-value</th>
<th>KN</th>
<th>KP</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN</td>
<td>-</td>
<td>0.022*</td>
<td>0.680</td>
<td>0.797</td>
<td>0.999</td>
<td>1.000</td>
</tr>
<tr>
<td>KP</td>
<td>-</td>
<td>-</td>
<td>0.380</td>
<td>0.278</td>
<td>0.047*</td>
<td>0.037*</td>
</tr>
<tr>
<td>P1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
<td>0.863</td>
<td>0.811</td>
</tr>
<tr>
<td>P2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.936</td>
<td>0.901</td>
</tr>
<tr>
<td>P3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.000</td>
</tr>
<tr>
<td>P4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*p-value<0.05 is significant

Table 2, from which one can infer notable distinctions between the KN and KP groups. Consistent with research investigating the impact of administering chitosan from white shrimp shells on the thickness of the trabecular bone of female mice femurs following ovariectomy, the ovariectomized rat group demonstrated an average trabecular thickness value of 59.53 µm while the group of ovariectomy mice had a smaller average trabecular thickness value of 32.44 µm (Rizalah et al., 2016). This indicates that the trabecular thickness of the ovariectomy group will decrease after ovariectomy. These results are echoed by research that states it is projected that women will undergo a decrease of around 50% in trabecular bone and 30% in cortical bone over their lifespan, with around half of this decline happening within the first ten years after menopause (de Villiers, 2023). During menopause, the primary effect of low estrogen levels is heightened cellular activity, leading to both bone resorption and formation. However, there's an imbalance where bone formation doesn't adequately counteract bone loss, resulting in accelerated bone loss and structural alterations such as disarray, thinning, and breakage of bone trabeculae. These changes significantly heighten the risk of fractures, primarily attributed to intense bone remodeling (Gosset et al., 2021).
These results are supported by other studies that state that *Phaleria macrocarpa* fruit meat contains flavonoid compounds, as the highest antioxidant substance, and flavonoids *Phaleria macrocarpa* fruit extract not only has one type of flavonoid but has six kinds of flavonoids, which causes *Phaleria macrocarpa* fruit to have high antioxidant and phytoestrogen potential that is good for the body (Dumanauw et al., 2022; Maharani et al., 2021). Phytoestrogen chemicals exert their effects by attaching to their receptors, which can occur through either ER-dependent or ER-independent routes. However, the ER-dependent route directly triggers its actions by attaching to the Estrogen Receptor α (ER-α) (Mirza et al., 2021). Additionally, a study has found that immunohistochemistry reveals a greater expression of ERβ in trabecular bone compared to cortical bone (cortex). ERα was shown to have an inverse pattern, with higher levels observed in cortical bone compared to trabecular bone, as indicated by the same study. Studies have demonstrated a correlation between variations in ERα and ERβ genes and bone mass in people (Biasan-Lauber & Lang-Muritano, 2022).

b. Thickness of Intima-Media Aortic in Mice Menopausal Model

The results of the intima-media thickness of the aorta of mice *Mus musculus* are shown in figure 2 using the Hematoxylin-eosin (HE) method.

*Figure 2.* Examination of the thickness of the intima-media aorta of mice with the method of Hematoxylin-eosin
Table 3. One-Way ANOVA Test of Aortic Intima-media Thickness

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Mean (µm)</th>
<th>Standard Deviation (SD)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN</td>
<td>12.2500</td>
<td>2.21736</td>
<td></td>
</tr>
<tr>
<td>KP</td>
<td>49.9375</td>
<td>13.42786</td>
<td>0.000</td>
</tr>
<tr>
<td>P1</td>
<td>27.4375</td>
<td>8.71391</td>
<td></td>
</tr>
<tr>
<td>P2</td>
<td>24.0625</td>
<td>6.21951</td>
<td></td>
</tr>
<tr>
<td>P3</td>
<td>22.3750</td>
<td>7.25862</td>
<td></td>
</tr>
<tr>
<td>P4</td>
<td>14.1875</td>
<td>1.79554</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 illustrates that the maximum mean intima-media thickness is in the positive group of 49.9375±13.42786 and the negative group of 12.2500±2.21736, significant results were obtained between the KP and the KN, namely sig = 0.000 (sig<0.05). This study can mean that the thickness of the aortic intima-media in the KN and the KP (ovariectomy) a meaningfully dissimilar. The effect of decreasing estrogen can affect the profile of cholesterol in the blood, one of which is the occurrence of memory in LDL (Low-Density Lipoprotein) (Pratiwi & Damayanty, 2020). Increased LDL in the blood can penetrate the endothelium and then accumulate in the nucleus cells (Huff et al., 2021). LDL that enters the nucleus of a cell will bind to free radicals or local reactive oxygen species (ROS), resulting in its transformation into LDL oxidation (Ox-LDL) (Jebari-Benslaiman et al., 2022). Ox-LDL prompts endothelial cells and smooth muscle to produce monocyte chemoattractant protein 1 (MCP-1), leading to heightened recruitment of monocytes to the sub-endothelium. Upon reaching the sub endothelium via MCP-1, circulating monocytes transform into macrophages. These macrophages possess scavenger receptors, which identify and engulf Ox-LDL, transforming into foam cells (Goo, 2019). Foam cells generate Platelet-Derived Growth Factor (PDGF), this process causes the movement of smooth muscle cells from the middle layer of the blood vessel wall to the inner layer, leading to the thickening of the inner layer (Erizon & Karani, 2020).

Table 4. HSD Post Hoc Test Results on Intima-media Thickness Data of Aortic Mice Menopausal Model after One Way ANOVA Test

<table>
<thead>
<tr>
<th>p-value</th>
<th>KN</th>
<th>KP</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
</tr>
</thead>
<tbody>
<tr>
<td>KN</td>
<td></td>
<td>0.000*</td>
<td>0.105</td>
<td>0.298</td>
<td>0.456</td>
<td>0.999</td>
</tr>
<tr>
<td>KP</td>
<td>-</td>
<td></td>
<td>0.007*</td>
<td>0.002*</td>
<td>0.001*</td>
<td>0.000*</td>
</tr>
<tr>
<td>P1</td>
<td>-</td>
<td>-</td>
<td></td>
<td>0.988</td>
<td>0.933</td>
<td>0.197</td>
</tr>
<tr>
<td>P2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>1.000</td>
<td>0.482</td>
</tr>
<tr>
<td>P3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>0.666</td>
</tr>
<tr>
<td>P4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
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</tbody>
</table>

*p-value<0.05 is significant

Table 4 shows that the are significant distinctions between the treatment group (KP) and treatment groups (P1-P4). Specifically, there’s a noteworthy variance between KP and P1, with a p-value of 0.007, KP and P2 with a p-value of 0.002, KP and P3 with a p-value of 0.001, and KP and P4 with a p-value of 0.000. Hence, it can be concluded that administering doses of flavonoids from Phaleria macrocarpa fruit extract ranging from 3.75 mg/mice/day to 15 mg/mice/day has a significant impact.

Phaleria macrocarpa fruit contains flavonoids, where flavonoids have a function as antioxidants. Antioxidants found in Phaleria macrocarpa fruit can reduce free radicals and lipid peroxidase so that it can make macrophages carry out their functions as cells that transport fat normally and can keep lipid levels in the blood remain at normal limits (Rochmah, 2008). In addition, flavonoids work as antioxidants by donating or releasing hydrogen ions to free radicals to become more stable. This activity blocks the reaction of OX-LDL (Low-Density Lipoprotein Oxidation), thus way, inhibiting the accumulation of fat on the blood vessel walls. Antioxidants can convert free radicals into low reactivity, so there is no reaction with fat and...
there is no accumulation of foam cells (Athiroh & Permatasari, 2012). Flavonoids have antioxidant activity that can increase the synthesis of Nitric oxide (NO) in the endothelium. Synthesized NO will cause vasodilation in vascular smooth muscle and can lower blood pressure. Nitric oxide is known to be the main regulator of smooth muscle. NO is one of the relaxation factors. Decreased bioavailability of NO due to endothelial dysfunction of blood vessels (Sadik, & Saiful Bachri, 2021).

4. CONCLUSION

The flavonoid extract from Phaleria macrocarpa fruit shows promise as a beneficial phytoestrogen and a potential alternative preventive measure against osteoporosis and atherosclerosis among menopausal women. Intervention with flavonoid extract from Phaleria macrocarpa fruit enhances the ratio of trabecular thickness and femoral bone cortex in a mice menopause model, particularly notable in intervention group 3 (P3) at a dosage of 11.25 mg/mice/day and intervention group 4 (P4) at a dosage of 15 mg/mice/day. Moreover, administering flavonoids from Phaleria macrocarpa fruit extract reduces the thickness of the intima-media aorta in the menopausal mice model, starting from the intervention group (P1) to P4, with dosages ranging from 3.75 to 15 mg/mice/day. Future studies could explore additional variables concerning other menopause conditions such as calcium and LDL levels.

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