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THE IMPACT OF FLAVONOIDS IN FRUIT EXTRACT FROM PHALERIA MACROCARPA ON CASPASE 3 EXPRESSION AND OSTEOCYTE COUNT IN FEMORAL BONE MICE USING MENOPAUSAL MODEL

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Abstract:
Women between the ages of 45 and 55 go through a phase known as menopause. Menstruation ceases for at least 12 months in those who experience menopause because of a decrease in ovarian function that lowers estrogen levels. Reduced estrogen levels can cause poor fat metabolism, which can lead to osteoporosis and atherosclerosis. The purpose of this study was to demonstrate the effect of a flavonoid extract obtained from
P. macrocarpa on the diameter expansion of the aorta and the rise in the number of osteocytes in the femoral bone of menopausal mice. This study utilized a genuine experimental laboratory setting with a research design of Randomized Post Test Only Control Group setting. Using 32 female mice divided into 6 groups: K- (without ovariectomy and flavonoid extract P.macrocarpa), K + (ovariectomy without treatment), P1 (ovariectomy + dose 3.75 mg/mice/day), P2 (ovariectomy + dose 7.5 mg /mice/day), P3 (ovariectomy + dose 11.25 mg/mice/day), and P4 (ovariectomy + dose 15 mg/mice/day). Administration of flavonoid extract P.macrocarpa was carried out for 14 days. Examination of Caspase 3 expression using the Immunofluorescence Data analysis method using SPSS 27.0. The results showed that in a post-hoc test, namely the administration of P.macrocarpa flavonoid extract at a dose of 11.25 mg/mice/day and 15 mg/mice/day showed that it could increase the number of osteocytes of the femoral bone of mice model of menopause. Based on the findings, it can be inferred that administering P.macrocarpa extract has a notable effect. Giving flavonoids from Phaleria macrocarpa fruit extract can reduce the expression of Caspase 3 and increase the number of osteocytes in the femoral bone of menopausal mice

Keywords: Menopause, *P.macrocarpa*, Caspase 3, Number of osteocytes

1. Introduction:

A physiological condition that affects women is menopause. Menstruation stops continuously for a year at the menopause, which is caused by an irreversible decrease in ovarian follicular activity. (WHO, 2022; Zhu et al., 2020). The WHO states that women typically go through menopause between the ages of 45 and 55. Early menopausal women often age less than 40 years, and their reasons might range from immune system disorders to chromosomal abnormalities or other undiscovered factors (WHO, 2022). The number of menopausal women in Indonesia increased from 15.8 million people in 2017 to 30.3 million people in 2020 (BPS, 2017). This indicates that in Indonesia more and more women are menopausal. Where menopause is a state where the beginning of the onset of the disease (Macpherson & Quinton, 2022).

Hypoestrogenemia, or low estrogen, is the outcome of ovarian follicular failure. Hypoestrogens are linked to abnormalities in fat metabolism that increase the risk of lipid peroxidation, which raises the risk of atherosclerosis and cardiovascular disease (CVD) (Newson, 2018). Moreover, hypoestrogens have the ability to expedite the breakdown of bone tissue and interfere with the natural process of bone regeneration, resulting in the onset of osteoporosis (Juwita et al., 2021). The incidence rate of CVD caused by atherosclerosis or so-called *Therosclerotic Cardiovascular Disease* (ASCVD) in women with an average age of 69 years in Canada from April 1, 2002 to March 31, 2018 was 432,300 from 1,042,621 (Hopper et al., 2021). In Indonesia, the prevalence of osteoporosis is significant, with 2 out of every 5 women being at an elevated risk of developing the condition (Juwita et al., 2021).

Due to a decrease in estrogen, menopausal women may have hypercholesterolemia, which raises low density lipoprotein (LDL) levels (Masyithah Pratiwi and dayanty, 2020). Oxidation of LDL occurs when LDL attaches to reactive oxygen species (ROS) (Ox-LDL). An ongoing rise in ROS causes tissue damage or oxidative stress, including endothelial dysfunction and a reduction in nitrite (NO) oxidation (Javadifar et al., 2021; Sargowo, 2015). An increase in LDL in the subendothelium is the cause of the increase in monocyte recruitment to the tunica intima and their dedifferentiation into macrophages (Javadifar et al., 2021). Ox-LDL can be recognized by scavengers in macrophages, who then absorb it into foam cells. in order for the mass of foam cells to develop into atheromatous plaques. Atherosclerosis is brought on by a rise in atheromatous plaques, which thicken the tunica intima media and reduce the aorta's diameter. (Khatana et al., 2020; Sargowo, 2015). Another impact of decreasing estrogen is that osteocytes are not able to trigger an adequate response to mechanical stress so that there is a decrease in mechanosensors in osteocytes and resulting in a decrease in the number of osteocyte cells (Cao et al., 2020; Kantola & Rantala, 2019). Osteocytes play a crucial function in maintaining the balance of calcium and phosphate in the body. Additionally, they serve as the main instigators and facilitators of bone remodeling by coordinating and directing the actions of osteoclasts and osteoblasts (Föger-Samwald et al., 2020).

Menopausal women are among those who need hormone replacement therapy (HRT) for hormonal problems, which include low estrogen levels (Khoudary et al., 2020). Hormone replacement therapy (HRT) has the capacity to decelerate the process of fat buildup and atherosclerosis in women experiencing menopause. In addition, HRT has a function to reduce the risk of fractures and prevent or treat osteoporosis in menopausal women (de Villiers, 2024; Gambacciani et al., 2018; Women Health Concern, 2021). However, due to long-term side effects that include an increased risk of stroke, lipid metabolism, pulmonary embolism, breast and uterine cancer, vaginal bleeding, and reduced liver function, HRT in postmenopausal women is still debatable when it comes to preventing ASCD and osteoporosis (Goldštajn et al., 2023; Nayak et al., 2022; Yousefzadeh et al., 2020).

Therefore, it is imperative to investigate alternative preventative methods, especially with regard to natural compounds that operate similarly to estrogen and have negligible side effects. A subset of these compounds are called phytoestrogens. Phenolic or polyphenolic phytochemicals are the chemical counterparts of phytoestrogens. In the realm of plants, it is the biggest and most prevalent phytochemical category (Bacciottini et al., 2007). One of the

phytoestrogens is the flavonoid extract *P.macrocarpa* (crown of gods) which is widely found in nature, especially in Indonesia (Ahmad et al., 2023). Flavonoids in *P.macrocarpa* fruit have potential as anti-microbial, anti-bacterial, antifungal, anti-allergic, antioxidant and vasodilator (Fitriana et al., 2023). In the flesh of *P.macrocarpa* there are six kinds of flavonoid compounds. 70% ethanol extract of the crown of god fruit has the largest relative flavonoid content of 45.734 µg/mg (Maharani & Sutrisno, 2021). By attaching to oestrogen receptors, phytoestrogens in plants can replicate or alter the effects of endogenous oestrogens. The endogenous estrogen is 17β-estradiol, mainly by binding to the ER. Phytoestrogens have an impact on oestrogen receptors, but they can also have antioxidant properties (Forslund & Anderson, 2017; Hasanah et al., 2020; Kuhnle et al., 2009).

The purpose of this work is to investigate how Phaleria macrocarpa affects the expression of caspase 3, the thickness of the intima-media, and the aortic diameter in menopausal model mice. Flavonoids found in Phaleria macrocarpa have the ability to affect caspase 3 expression activity. One of flavonoids' key functions is that they may donate hydrogen and scavenge radicals. Another role of flavonoids is to prevent zymogen division. Either directly reducing Caspase 3 or indirectly inhibiting Caspase 9, the starter caspase, can reduce Caspase 3 activity. thereby blocking the zymogen's cleavage, which would have activated Caspase 3, the executioner caspase. Furthermore, it functions by blocking the OX-LDL reaction, which stops fat from accumulating. of the blood artery walls, which results in a reduction in the aortic diameter's narrowing and the thickness of the intima-media.

This work is new in that it examines the effects of feeding P. macrocarpa fruit extract, where separated flavonoids may be identified as one of the phytoestrogens' particular active ingredients. There have been positive findings from other research on the benefits of P. macrocarpa fruit extract flavonoids on conditions including diabetes and endometriosis, but no studies on menopause have been conducted to far. This study aimed to investigate the effects of flavonoids from P. macrocarpa (god's crown) extract on caspase 3 expression and the number of osteocytes in the menopausal mice's femur bone

2. Material and Method

This study employed a real experimental laboratory design with a research design of Control group design with only one random post-test. Mice (Mus musculus) in a menopausal model served as the sample. The East Java Province's Farma Veterinary Center (Pusvetma) provided the healthy mice. A random selection of mice will be made to comprise each group. 32 mice total will be utilized in this investigation, 30 of which will be split into 6 treatment groups and 2 heads for FSH monitoring, with 1 tail in the treatment group and 1 tail in the control group. The purpose of FSH checking is to determine whether or not mice have entered menopause; if FSH levels in the treatment group are higher than in the control group, then mice may have entered menopause. (Rodríguez-Landa, 2022). The Anatomical Pathology Laboratory of the Faculty of Medicine, Universitas Brawijaya is used for aortic diameter examination, and the Embryology Laboratory, Faculty of Veterinary Medicine, Universitas Airlangga is used for maintaining mice (Mus musculus), producing menopausal model mice, and administering flavonoid treatment of P. macrocarpa extract..

In this study, mice were divided into six groups: K- (control group), K + (positive group), P1 (ovariectomy and flavonoid extract P. macrocarpa dose 3.75 mg/mice/day), P2 (ovariectomy and flavonoid extract P. macrocarpa dose 7.5 mg/mice/day), P3 (ovariectomy and flavonoid extract P. macrocarpa dose 11.25 mg/mice/day), and P4 (ovariectomy and flavonoid extract P. macrocarpa dose 11.25 mg/mice/day) (Maharani & Sutrisno, 2021). Eighth-day divariectomy mice were given a 28-day recuperation time. Bilateral ovariectomy, also known as ovariectomy, is the removal of both ovaries from a mouse. Two mice were surgically removed for an FSH investigation following the recuperation time. Following an increase in FSH, mice were treated with a P. macrocarpa flavonoid extract for 14 days at a level determined by the group. The menopause model is based on bilateral ovariektomi. ekstrak Phaleria macrocarpa dengan

menggunakan sonde lambung selama 14 hari. The organ in use is the descending thoracic aorta. The Caspase 3 expression is analyzed using the Immunofluorescence method.

The P. macrocarpa fruit is processed in the Batu materia medica laboratory after being received from Batu City, East Java. The peel of the fruit was utilized for this investigation, and the mature flesh—which is where the seed is extracted—is distinguished by the fruit's maroon skin. Following mashing and soaking in 96% ethanol for approximately half an hour, the P. macrocarpa fruit is left to settle for five days. Next, use a buncher funnel to filter the marinade in order to obtain maserat.. Maserat from the god's crown (P. macrocarpa), which still includes ethanol solvent, is then heated to 60°C and let to evaporate using an evaporator for eight hours to produce a thick extract. After that, it is fractionated using n-hexane and n-butanol partitions in a liquid-liquid method. (Maharani et al., 2021).

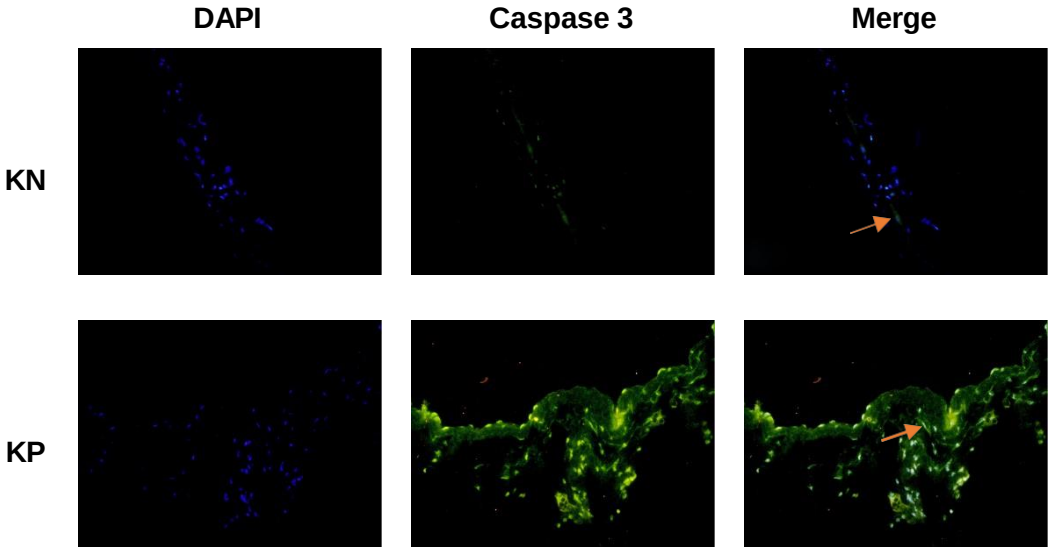
The following step is termination for organ harvesting and preparation after 14 days of P. macrocarpa extract administration. Mice are given ketamine injections before to surgery. Once the descending thoracic aorta and posterior extremity had been dissected and sliced to remove the femur bone and posterior extremity of the mice, both of them were placed in separate vials containing 10% formalin for a period of seven to twenty-four hours, making sure that the organs were completely immersed. The organ is then severed within the aorta and left to decalcify in the femoral bone using an EDTA solution, which takes seven to fourteen days. (Dewi, Y et al., 2020; Liu et al., 2017). By using Hematoxylin-Eosin (HE) staining, the number of femoral osteocytes may be determined. We shall cut each organ to a thickness of 3–4 μm. Using Aperio CS2 Leica, the colored organs were scanned, and ImageScopex64 was used to calculate the results. The results from the comparison of the femoral osteocyte count and aorta diameter in menopausal mice model between the treatment group and the control group were statistically analyzed using the SPSS 27.0 program for Windows. Shapiro-Wilk test for normality, Leven test technique for homogeneity, One-way ANOVA test for homogeneity, and Tukey HSD method for post hoc testing..

Research actions are in accordance with the applicable research code of ethics and have been approved by the Faculty of Medicine, Brawijaya University, Malang, Indonesia, by issuing an ethics number: 26/EC/KEPK–S2/01/2024 dan 108/EC/ KEPK-S2/05/2024

3. Result

3.1. Expression of Caspasee 3 of mice Menopausal Model

The results of examining Caspase 3 expression in the aorta of Mus musculus mice are shown in Figure 5.1 using the immunofluorescence (IF) method using primary antibody (anti Caspase FITC) Santa Cruz no: sc-7272



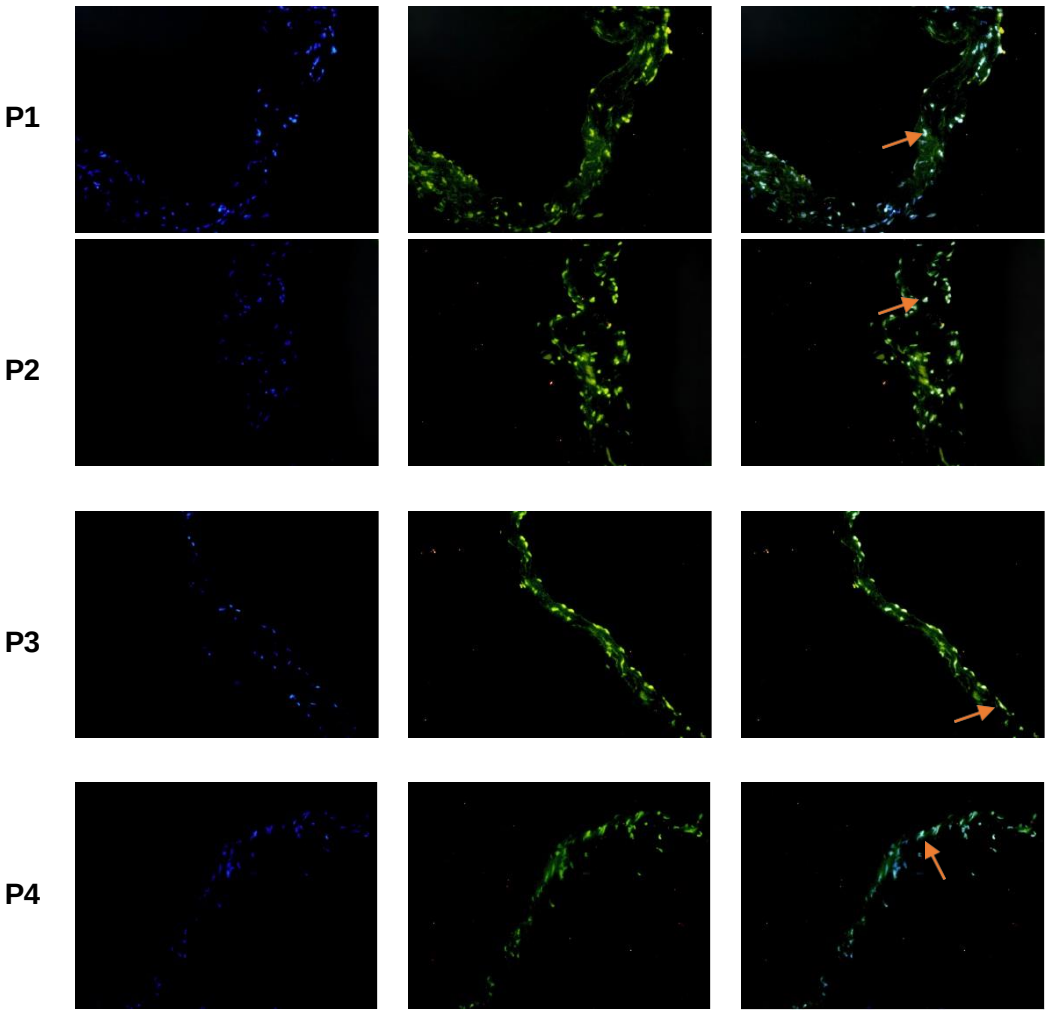


Figure 1 Examination of Mus musculus aortic Caspase 3 expression in the menopause model

Description: Aortic tissue subjected to immunofluorescence staining at 400x magnification. (A) control group (KN) of healthy mice with an average expression of Caspase 3 (5.6540 ± 5.17206), (B) positive group (KP) of ovariectomized mice with an average expression of Caspase 3 (29.6387 ± 4 .32516), (C) group P1 of ovariectomized mice + dose of Phaleria macrocarpa extract 3.75 mg/mouse/day with average expression of Caspase 3 (23.4183±3.58034), (D) group P2 of ovariectomized mice + dose Phaleria macrocarpa extract 7.5 mg/mouse/day with an average Caspase 3 (18.4718±3.96945), (E) P3 group of ovariectomized mice + dose of Phaleria macrocarpa extract 11.25 mg/mouse/day with an average average expression of Caspase 3 (12.0788 ± 3.07234), (F) group P4 of ovariectomized mice + dose of Phaleria macrocarpa extract 15 mg/mouse/day with average expression of Caspase 3 (8.7865 ± 3.86779). (↑) Caspase 3 expression

The normality test and homogeneity test are parametric prerequisite tests before carrying out the One-way ANOVA test. Data can be said to be normal and homogeneous if the p-value is (>0.05). Table 5.1 explains that in the normality test using the Shapiro-Wilk test because the number of samples was less than 50, the result was a p-value of 0.665 (p>0.05) and the homogeneity test using the Lavene test resulted in a p-value of 0.797 (p >0.05). So it can be concluded that from these two tests the aortic Caspase 3 data is normally distributed and the data is homogeneous. After the data is declared normally distributed and homogeneous, the data can be continued for the One-way ANOVA test. Where the One-way ANOVA test is a parametric test carried out to test the research hypothesis.

Table 1 Normality and homogeneity test results on Caspase 3 Aorta Mus musculus Menopause Model

Treatment Group	N	*P-Value Shapiro-Wilk*	Data distribution	*P-Value Levene test*	Homogenity
KN	4	0,716			
KP	4	0,281			
P1	4	0,061			
P2	4	0,603	Normal	0,797	Homogen
P3	4	0,065			
P4	4	0,302			
Total	24	0,665			

Note: Data is normally distributed and homogeneous if the p-Value is >0.05.

The next stage is to see whether there is a significant difference in the average of each group using the One-Way ANOVA test. The results of the One-way ANOVA test can be seen in table 2

Table 2 One-Way ANOVA Test Caspase 3 Aorta Mus musculus Menopause Model

Treatmen Group	Mean ± SD (Eskpresi Caspase 3 (au))	*p- Value One-way ANOVA*
KN	5,6540±5,17206[a]	
KP	29,6387±4,32516[d]	
P1	23,4183±3,58034[cd]	0,000
P2	18,4718±3,96945[bc]	
P3	12,0788±3,07234[ab]	
P4	8,7865±3,86779[a]	

Note: p-Value 0.05 means there is a significant difference. SD: standard deviation; au: arbitrary units

Table 2 shows that the average value of aortic Caspase 3 expression in the positive control group (KP) (29.6387 ± 4.32516) is higher than the negative control group (KN) (5.6540 ± 5.17206). In the treatment group given 4 different doses, the average expression of Caspase 3 was lower compared to the group of ovariectomized mice (KP). The P1 group given 3.75 mg Phaleria macrocarpa flavonoid extract/mouse/day (23.4183±3.58034) was lower than the KP group. Group P2 given 7.5 mg Phaleria macrocarpa flavonoid extract/mouse/day (18.4718±3.96945) was lower than group P1. Group P3 given 11.25 mg Phaleria macrocarpa flavonoid extract/mouse/day (12.0788±3.07234) was lower than group P2. Meanwhile, the P4 group with 15 mg Phaleria macrocarpa flavonoid extract/mouse/day (8.7865±3.86779) was lower than the P3 group and was close to the value of the KN group, namely without ovariectomy or administration of Phaleria macrocarpa flavonoid extract.

In table 1 it is stated that the results of the One-Way ANOVA test are p=0.000, which means that there are significant differences in all research groups. However, these results cannot determine which groups have significant differences between the 6 research groups. Therefore, a post hoc test was then carried out using the Tukey Honestly Significant Difference test. The results of the post hoc test can be seen in the table 3

Table 3 Results of Post hoc HSD Test on Caspase 3 Aorta of Mus musculus Menopausal Model given Flavonoids from Phaleria macrocarpa Fruit Extract

P-value	KN	KP	P1	P2	P3	P4
KN		,000*	,000*	,003*	0,267	,878
KP			,297	,011*	,000*	,000*
P1				,553	,010*	,001*
P2					,271	,033*

P3	,854

*p-value<0.05 is significant

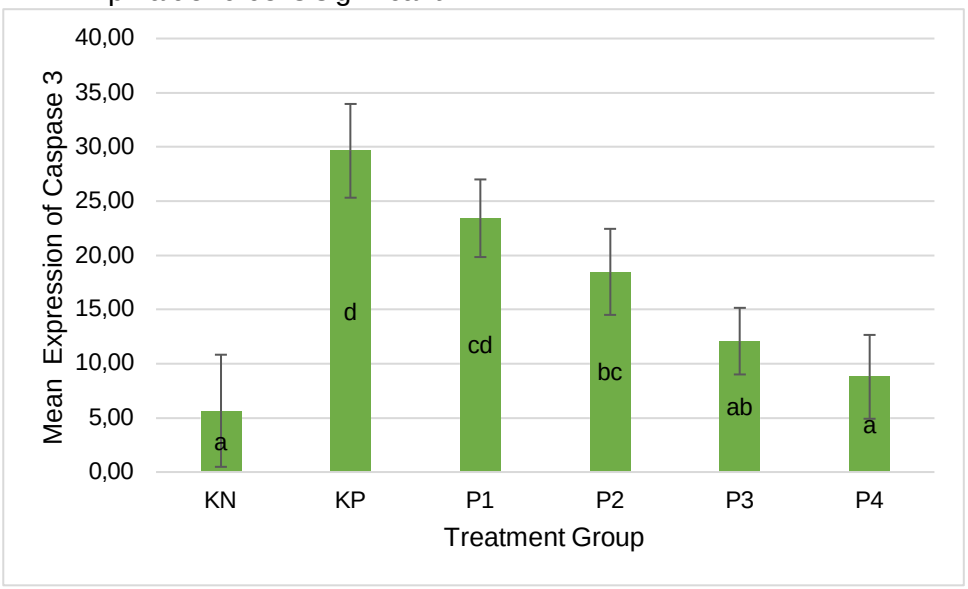


Figure 2 Average Histogram of the Effect of Phaleria macrocarpa Fruit Extract Flavonoids on Caspase 3 Expression

Description: (KN) normal mice. (KP) ovariectomized mice + without administration of Phaleria macrocarpa flavonoid extract. (P1) ovariectomy + Phaleria macrocarpa flavonoid extract 3.75 mg/mouse/day. (P2) ovariectomy + Phaleria macrocarpa flavonoid extract 7.5 mg/mouse/day. (P3) ovariectomy + Phaleria macrocarpa flavonoid extract 11.25 mg/mouse/day. (P4) ovariectomy + Phaleria macrocarpa flavonoid extract 15 mg/mouse/day. If it contains different letters (a, b, c or d) then there is a significant difference (p-value <0.05), whereas if it contains the same letter (a with ab, b with ab and bc, or d with cd) then there is no there is a significant difference (p-value >0.05).

The post hoc test is the final test that explains which groups have significant mean differences by looking at the p-value <0.05. The KN group had a significant average difference with the KP group (p=0.000), namely the group that underwent ovariectomy but was not given flavonoids from Phaleria macrocarpa fruit extract. This shows that Caspase 3 expression in the ovariectomy group (KP) (29.6387 ± 4.32516) was higher compared to the control group (KN) (5.6540 ± 5.17206). Apart from that, KN also had a significant average difference with treatment group 1 (P1) with a significant value of p=0.000, namely the group that underwent ovariectomy and was given a dose of Phaleria macrocarpa extract at a dose of 3.75 mg/mouse/day, but P1 did not have an average difference. -average is significant in the positive group (KP). This shows that the average expression of Caspase 3 in the P1 group (23.4183 ± 3.58034) is close to the average value in the KP group (29.6387 ± 4.32516). The KN group had a significant mean with the P2 group (18.4718 ± 3.96945) and the P2 group had a significant mean with the KP group (0.011).

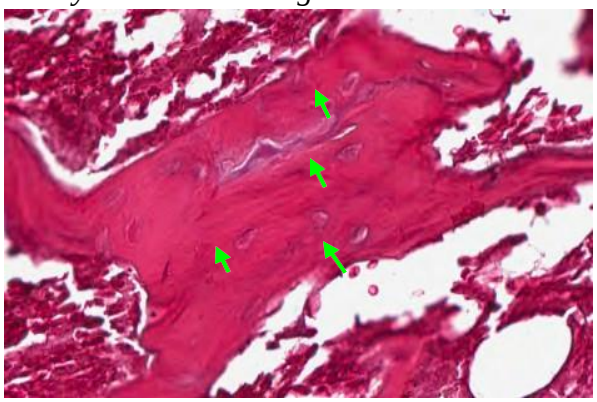
The positive control group (KP) has a significant average difference with treatment group 2 (P2) with a significant value of p=0.011, while in treatment group 3 (P3) with a significant value of p=0.000 and treatment group 4 (P4) with a significant value of significant p=0.000. This shows that the expression of Caspase 3 in the aorta of mice treated with flavonoid extract from Phaleria macrocarpa was lower compared to the group of ovariectomized mice. Seen in table 5.2, the average expression of Caspase 3 in the treatment group (P2) (18.4718 ± 3.96945) with a dose of 7.5 mg/mouse/day was lower compared to the ovariectomized mice group (29.6387 ± 4.32516). The average of the P3 group (12.0788 ± 3.07234) with a dose of 11.25 mg/mouse/day and P4 (8.7865 ± 3.86779) with a dose of 15 mg/mouse/day is lower than the mouse group ovariectomy

The treatment group (P1) had a significant average difference with groups P3 (p=0.010) and P4 (p=0.001), meaning that the average Caspase 3 in the P3 group (12.0788±3.07234) had an average of The mean was significantly lower than the P1 group (23.4183±3.58034), and the Caspase 3 mean of the P3 group (12.0788±3.07234) was close to the KN mean value (5.6540±5.17206) and group P2 (18.4718±3.96945). Likewise, the P4 group had a significantly lower average than the P1 group, and the average Caspase 3 in the P4 group was close to the value of the KN and P3 groups.

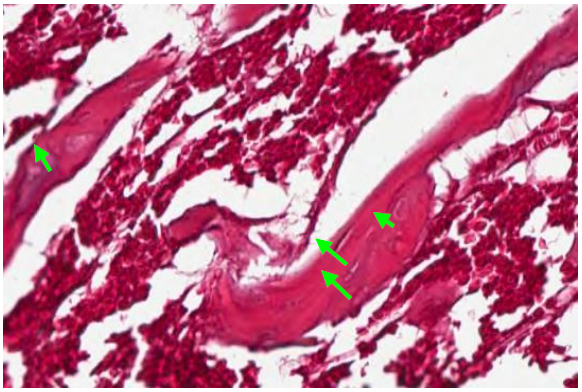
The treatment group (P1) had a significant average difference with groups P3 (p=0.010) and P4 (p=0.001), meaning that the average Caspase 3 in the P3 group (12.0788±3.07234) had an average of The mean was significantly lower than the P1 group (23.4183±3.58034), and the Caspase 3 mean of the P3 group (12.0788±3.07234) was close to the KN mean value (5.6540±5.17206) and group P2 (18.4718±3.96945). Likewise, the P4 group had a significantly lower average than the P1 group, and the average Caspase 3 in the P4 group was close to the value of the KN and P3 groups.

3.2. Number of femoral osteocytes of mice Menopausal Model

Results of staining the number of femoral osteocytes of mice menopausal model with *Hematoxylin-Eosin staining.*



(A)



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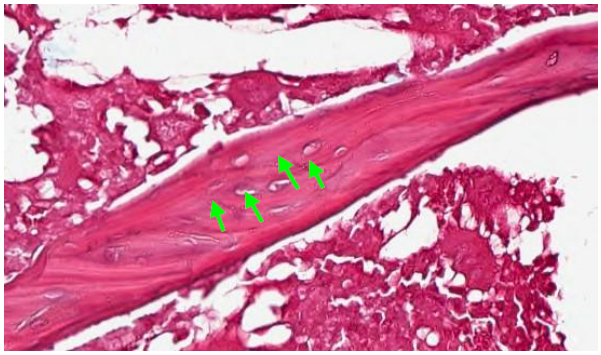

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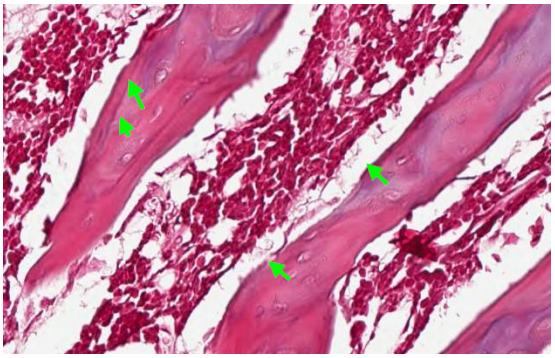

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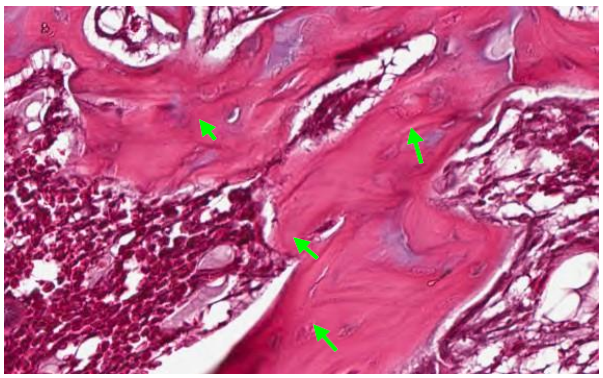
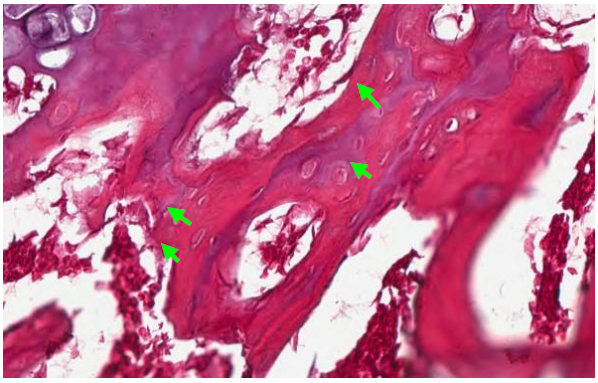


(E)



(F)

Figure 3. Hispathology of the number of mice osteocyte cells . Image with 200x magnification with HE coloring. **(A)** K- (without ovariectomy and without administration of flavonoid extract *P.macrocarpa*), **(B)** K+ (ovariectomy and without administration of flavonoid extract *P.macrocarpa*), **(C)** P1 (ovariectomy and administration of flavonoid extract *P.macrocarpa* dose of 3.75 mg/mice/day), **(D)** P2 (ovariectomy and administration of flavonoid extract *P.macrocarpa* dose 7.5 mg/mice/day), **(E)** P3 (ovariectomy and administration of flavonoid extract P.macrocarpa dose 11.25 mg/mice/day), **(F)** P4 (ovariectomy and administration of flavonoid extract *P.macrocarpa* dose 11.25 mg/mice/day).

The one-way ANOVA test on the average data of the number of osteocytes of femoral bone in a menopausal mouse model revealed significant differences in all groups of observation samples. The detailed results are given in the table below.

Table 4. One-Way ANOVA Test of Number of Osteocytes in Mice Menopausal Model

Treatment Group	Average ± SD (Number of osteocytes (cells))	*p-value one-way ANOVA*
K-	42.040 ± 10.029	
K+	22.960 ± 3.398	
P1	27.400 ± 6.310	0.001
P2	32.560 ± 7.045	
P3	37.240 ± 7.714	
P4	42.080 ± 6.577	

The one-way ANOVA test conducted on the mean number of osteocyte cells in the femoral bone of the menopausal model produced a statistically significant result, with a p-value of 0.001 ($p < 0.05$). These data indicate that there were significant differences among the observed groups. This indicates that there were also notable variances within each treatment group (P1-P4) after administering varying amounts of flavonoids from *P.macrocarpa* fruit extract for a duration of 14 days.

This study examined the quantity of osteocytes, a crucial type of cells found in bones. The findings of this study revealed that the negative control group (K-) had a greater quantity of osteocytes compared to the positive control group (K+), in which mice underwent ovariectomy treatment to simulate a model of menopausal mice. The data presented in table 3 shows that the average number of osteocytes in group K- is 42,040 cells, while in group K+ it decreased to 22.960 cells. Furthermore, the data analysis confirms that there is a significant difference between group K- and group K+, with a significance value of $p = 0.003$ ($p < 0.05$). The study determined that performing ovariectomy on mice to induce menopause can lead to a reduction in the quantity of osteocyte cells in the femoral bone. The findings of this study align with previous research that investigated the quantity of osteocytes in the tibia bone of rats that underwent divariectomy. It was observed that the average number of osteocytes in rats who underwent divariectomy without therapy was decreased compared to normal mice (Pratiwi, 2019).

After menopause, a hormonal insufficiency of estrogen leads to a decrease in the number of osteocyte cells, which are essential for maintaining bone equilibrium. The decline in the quantity of primordial follicles in females results in a reduction in ovulation. As a result, the corpus luteum reduces its synthesis of estrogen. As a result, this impairs the osteocytes' capacity to effectively react to mechanical strain. As a result, there is a decrease in mechanosensors in osteocytes and a reduction in the number of osteocyte cells (Cao et al., 2020; Kantola & Rantala, 2019). In line with other studies that the number of osteocytes that can live in the human femur bone decreases with age, the proportion of osteocyte cells that can live decreases from 88% at the age of 10-29 years to 58% at the age of 70-89 years, that is, the postmenopausal age in women (Kobayashi et al., 2015). In another study, it was also explained that osteocyte cell apoptosis and canaliculi tissue damage will increase progressively with age in mice, thereby reducing connectivity between osteocytes and other tissues (Bonewald, 2011). In addition to their crucial role in regulating calcium and phosphate levels, osteocytes also play a vital role in initiating and driving bone remodeling. They accomplish this by interacting with and orchestrating the creation and activity of osteoblasts and osteoclasts (Föger-Samwald et al., 2020). Thus, this work demonstrates a noticeable reduction in the quantity of osteocytes within the femur bone of mice subjected to ovariectomy, which serves as a menopausal model. Additionally, therapy is administered to enhance the quantity of osteocytes. Nevertheless, the outcomes of the One-way ANOVA test were inconclusive in identifying the specific groups that exhibited significant differences among the six observation groups. Consequently, the Post Hoc Test was conducted for multiple comparisons using the HSD test. The comprehensive results may be found in the table below*:*

Table 4. Post hoc HSD Test of Number of Osteocytes in Mice Menopausal Model

p-value	**K-**	**K+**	**P1**	**P2**	**P3**	**P4**
K-		0.003*	0.035*	0.318	0.890	1.000
K+			0.918	0.306	0.042*	0.003*
P1				0.857	0.281	0.035*
P2					0.900	0.314
P3						0.887
P4						

*p-value<0.05 is significant

This study has shown the effects of providing flavonoids from P.macrocarpa fruit extract on the number of osteocytes in the femoral bone of mice with menopause. The P1 treatment group, which was administered a dosage of 3.75 mg/mice/day, exhibited a similar number of osteocytes as the positive control group (K+). Nevertheless, there has been a rise in the quantity of osteocyte cells, with an average of 22.960 cells in the K+ group and 27.400 cells in the P1 group. The P1 group exhibits a statistically significant difference compared to the K- group, with a significance level of $p = 0.035$ ($p < 0.05$). The experimental group 2 (P2) was administered a dosage of 7.5 mg/mice/day, resulting in a substantial increase in the mean count of osteocyte cells, reaching 32.560 cells. The K+ group exhibited a significant rise in the quantity of osteocytes compared to treatment group 3 (P3) and treatment group 4 (P4), which were administered doses of 11.25mg/mice/day and 15mg/mice/day, respectively. The P4 group is nearly equivalent to the normal group (K-) with an average osteocyte cell count of 42.080 cells. Based on this study, it can be inferred that the effective and significant dose of flavonoids from *P.macrocarpa* fruit extract for raising the number of osteocytes in the femoral bone of menopausal mice is either 11.25 mg/mice/day or 15 mg/mice/day.

4. Discussion

Phaleria macrocarpa, often known as the Mahkota dewa, has flavonoid content that can affect caspase 3 expression. One of flavonoid's most important properties is that it may act as a hydrogen-fixing agent and a radial oxidizer. Because of this, flavonoids can increase endogenous antioksidan and inhibit the production of ROS and selenium later on. Flavonoids that include radical-forming compounds can inhibit the oxidation of low-density lipoprotein (LDL). (Mahmoud *et al.*, 2019). In addition, another study clarifies that zymogen replacement is necessary for Caspase 3 activities. When zymogen is not broken down by caspase initiator after the presence of apoptotic signals, Caspase 3 functions as an executor and does not become active. Another function of flavonoids is to inhibit zymogen synthesis. Reducing the activity of Caspase 3 can play an indirect or direct role by inhibiting the initiator caspase, namely Caspace 9, disseminating zymogens to produce the activation of the invasive caspaze, Caspaze 3.(White *et al.*, 2012)

Another study also examined the number of osteocytes in menopausal model animals with the average result that the number of osteocytes of ovariectomy rats that were not treated was also lower than that of ovariectomy mice that were given treatment. This proved that the number of osteocyte cells that were divariectomy was lower than normal mice, and if hypoestrogen was not given therapy for a long time it would reduce the number of osteocytes as in untreated ovariectomy mice (Pratiwi, 2019). A separate study investigated the impact of Moringa leaf extract on the quantity of osteocyte cells. The study revealed a discrepancy in the quantity of osteocyte cells and the dimensions of the mandibular bone matrix in wistar rats between the control group and the group administered with Moringa leaf ethanol extract (Moringa oleifera). Osteocyte cell count and bone matrix have a gradual rise till day 28. The study also reported that the flavonoid chemicals found in Moringa leaves (Moringa oleifera) aid in calcium metabolism by reducing bone resorption (Chairunas et al., 2018). Consistent with this research, the administration of flavonoid extracts from *P.macrocarpa* fruit significantly augmented the quantity of osteocyte cells in mice experiencing menopause.

Osteocyte cells are essential for maintaining the equilibrium of bone remodelling.In addition to regulating calcium and phosphate levels, osteocytes also serve as key initiators and drivers of bone remodeling. They communicate with and coordinate the actions of osteoblasts and osteoclasts, influencing their formation and activity (Föger-Samwald et al., 2020). Bones are not static structures but are metabolically active in maintaining mineral homeostasis; Osteoporosis is constantly undergoing changes in response to a variety of influences, including mechanical stress, as well as hormonal and immunological stresses (Birbrair et al., 2017). Plant foods that we eat on a daily basis contain a class of naturally occurring chemicals called flavonoids, which have different phenolic structures. Due to its strong ability to fight free radicals, antioxidant activity, and anti-resorptive properties, flavonoids offer a variety of advantageous effects (Ramesh et al., 2021). Flavonoids function as phytoestrogens. Additional research also suggests that phytoestrogens are plant-derived dietary components with similar structures to 17-β-estradiol, the main female sex hormone. Phytoestrogens can induce estrogenic effects by attaching to oestrogen receptors as a result of their structural similarity to E2 (Rietjens, 2017). This suggests that flavonoid *P.macrocarpa* fruit extract can be an alternative prevention of reduced osteocyte cells in bones during menopause

5. Conclusion

Phenols A botanical medicine called P. macrocarpa fruit extract (containing phytoestrogens) may be used in place of lowering the risk of osteoporosis and atherosclerosis in women going through menopause. The aortic diameter and femoral bone osteocyte count may rise as a result of treatment group 3 (P3) receiving a dose of 11.25 mg/mice/day and

group 4 receiving a dose of 15 mg/mice/day. When menopausal model mice were given flavonoid extract from Phaleria macrocarpa, namely in groups P2 to P4, at a dosage of 7.5 to 15 mg/mouse/day, the expression of caspase 3 in the aorta was reduced in comparison to ovariectomized mice

6. Conflist of Interest

No conflict of interest

7. Author Contribution

All authors contributed to the process of preparing this scientific article

8. References

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