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The effectiveness of Kersen Leaf extract (*Muntingia calabura* L.) on mice (*Mus musculus* L.) spermatozoa after D-Galactose induction

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Abstract
Purpose: This study aimed to test the effectiveness of kersen leaf extract (*Muntingia calabura* L.) on mouse spermatozoa (*Mus musculus* L.) after d-galactose induction.
Research Methodology: This study was conducted between September and October 2023 at the Zoology Laboratory of the University of Lampung. This study used a Completely Randomized Design (CRD) including five treatment groups. Each treatment included five mice: positive control (K0), negative control (K-) induced D-galactose 150 mg/kg body weight, treatment 1 (P1)-induced D-galactose 150 mg/kg body weight, Kersen leaf extract 35 mg/kg body weight, (P2) induced D-galactose 150 mg/kg body weight, and Kersen leaf extract 70 mg/kg body weight, and (P3) induced D-galactose 150 mg/kg body weight and Kersen leaf extract 105 mg/kg body weight for 35 days. Normal and homogeneous data were analyzed using analysis of variance (ANOVA) to determine whether the treatment's effect was significant. Differences were considered statistically significant at $p < 0.05$. Duncan's multiple interval post hoc test (=5%) was used to identify the most effective treatment.
Results: Analysis of Variance (ANOVA) showed that the total, viability, motility, and morphology of normal spermatozoa increased after induction with Kersen leaf extract at a dose of 105 mg/kg BB.
Limitations: This study provides readers with knowledge on natural antioxidants that prevent free radicals.
Contribution: This study provides readers with knowledge on natural antioxidants that prevent free radicals.
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1. Introduction

Premature aging is a phenomenon that occurs in many people who experience aging prematurely. During the aging process, organs undergo functional changes that lead to a decrease in the physiological effects of the organ system, resulting in a reduction in quality of life (Situmorang & Zulham, 2020). Free radicals are one of the many factors that contribute to premature aging. Increased reactive oxygen species (ROS) from free radicals damage sperm DNA by inducing apoptosis in spermatozoa cells and interfering with spermatogenesis (Awuy, Purwanto, & Mewo, 2021). Pituitary dysfunction caused by oxidative stress lowers serum testosterone levels and suppresses gonadotropin-releasing hormone (GnRH). Additionally, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) secretion decline (Ahangarpour, Oroojan, & Heidari, 2014).

Oxidative stress can cause plasma membrane damage and abnormal spermatozoa morphology due to the induction of lipid peroxidation. In addition to inducing lipid peroxidation, oxidative stress can also induce DNA damage, accelerate the apoptosis of germ epithelial cells, and reduce the number of spermatogenic cells (Permatasari & Widhiantara, 2017). Lipid peroxidation can trigger the formation and increase the levels of malondialdehyde (MDA), an indicator of oxidative stress. Apoptosis in spermatozoa is caused by the presence of free radical compounds, as the increasing number of ROS is the basis of damage to seminiferous tubules (Dewanto & Isnaeni, 2017).

Spermatogenesis is the process of spermatogonia formation into spermatozoa, which occurs in the seminiferous tubules of testes. Seminiferous tubules are lined by Sertoli cells and spermatogonia, which function to provide nutrients for immature sperm and produce the hormone *Androgen Binding Protein* (ABP) (Malini, Ratningsih, Fitriani, & Rahmi, 2020). The testes are vulnerable to oxidation and free radicals, which can cause problems in spermatogenesis and the spermatozoa membrane. Spermatogenic cell membranes are composed of unsaturated fatty acids that can result in lipid peroxidation, increased membrane fluidity, and inactivation of membrane bonds with enzymes and receptors owing to incoming free radicals and oxidative stress. These conditions will increase spermatozoa cell damage, decreased intracellular ATP, decrease spermatozoa viability, and cause sperm morphology to be damaged and loses of the capacitation and reaction ability of the acrosome spermatozoa (Sukmaningsih, Ermayanti, Wiratmini, & Sudatri, 2011). D-galactose administration in animal models can increase the production of ROS and advanced glycation end-products, thus triggering oxidative stress (Situmorang, 2021). Antioxidants, such as kersen leaves, are known to prevent this problem.

Kersen leaf extract showed a protective effect on D-galactose-induced spermatogenesis in male mice (Badami, 2017). D-galactose induction in animal models can increase the production of ROS and advanced glycation end-products, thus triggering oxidative stress (Situmorang, 2021). Based on the description above, it is very interesting to study the effect of kersen leaf extract (*Muntingia calabura* L.) on the total, viability, motility, and morphology of mouse spermatozoa after D-galactose induction.

2. Methodology

2.1 Study Design

This is a pure experimental study using a completely randomized design (CRD) and a post-test only control group strategy. 25 male wistar mice weighing between 25 and 35 g, aged 8–10 weeks, were housed with food, water, and sufficient air circulation. The mice were separated into five groups: control and treatment groups. The Health Research Ethics Committee, Faculty of Medicine, University of Lampung, has completely evaluated all procedures and issued approval number 3841/UN26.18/PP.05.02.00/2023.

2.2 Induction of D-Galactose

20 mice were given 150 mg/kg body weight D-Galactose orally for 35 days. According to Sulistyoningrum (2017), the appropriate dose for reducing sperm parameters is 100-200 mg/kg body weight. Induction of D-galactose increases free radical levels in the body, causing oxidative stress in mice and may hasten the aging process. To ensure that the samples were homogeneous, computations were performed using the body weights of the experimental animals. During the treatment, the health of the mice was monitored, including their daily behavior, hunger, and body weight.

2.3 Preparation Kersen Leaf Extract

Fresh kersen leaves were washed thoroughly with flowing water to remove any dirt on the leaves, aerated overnight, and placed in an oven at 37℃ for three days. After drying, the leaves were chopped into small pieces and mashed in a blender. Kersen leaf powder was macerated with distilled water for 7 days at a concentration of 100 g per 1 L of distilled water. Furthermore, the concentration process is carried out using a rotary evaporator with a temperature of 60℃to produce a thick extract and then dried in a desiccator to produce a dry extract which is used for experiments (Syabania, Pambudi, Wirasti, & Rahmatullah, 2021).

2.4 Kersen Leaf Extract Treatment

The treatment group of mice with induced D-galactose was administered therapy by giving kersen leaf extract at a dose of 35 mg/kg body weight (P1), 70 mg/kg body weight (P2), and 105 mg/kg body weight (P3). Kersen leaf extract was administered orally with a sonde daily at 9 a.m. Kersen leaf extract was used because kersen leaves contain bioactive substances that have been shown to reduce the effects of free radicals on cells (Khan, Mundasada, & Ramadas, 2015).

2.5 Sample Collecting

After the cauda epididymis was sliced and separated from the testes, the spermatozoa were extracted. Next, the cauda epididymis was placed in a dish with 0.9% NaCl and carefully compressed with tweezers to extract the liquid spermatozoa. The spermatozoa suspension was then diluted with 1 mL 0.9% NaCl and 1% eosin and examined under a microscope.

2.6 Statistical Analysis

The observational data were statistically examined. The analysis was performed using the SPSS program 25. The Shapiro-Wilk test was used to check the data for normality, followed by the Levene test for homogeneity. Homogenized data were examined using ANOVA. The most effective treatment was determined using Duncan's post-hoc (°C =5%) multiple interval test when $p < 0.05$.

3. Result and discussion

The results of observations on the total, viability, motility, and morphology of spermatozoa showed different results for each treatment. The results are presented in Table 1.

Table 1. The percentage of spermatozoa observation

Exp Group.	Average ± SD Mice Spermatozoa			
	Total (mill/mL)	Viability (%)	Motility (%)	Morphology (%)
K0	95.60 ± 41.34^{c}	54.00 ± 0.06^{c}	55.00 ± 0.05^{b}	52.00 ± 0.30^{b}
K-	51.20 ± 6.61^{a}	41.00 ± 0.06^{a}	40.00 ± 0.38^{a}	39.00 ± 0.79^{a}
P1	59.00 ± 10.53^{ab}	46.00 ± 0.01^{bc}	55.00 ± 0.10^{b}	53.00 ± 0.99^{b}
P2	69.80 ± 7.82^{abc}	48.00 ± 0.04^{bc}	55.00 ± 0.28^{b}	59.00 ± 0.37^{b}
P3	82.20 ± 6.53^{bc}	54.00 ± 0.06^{c}	65.00 ± 0.64^{c}	$63.00 \pm 0,44^{bc}$

*differences letter indicates results that are significantly different statistics

The results of microscopic observation of spermatozoa morphology in mice (*Mus musculus* L.) are presented in Figure 1.

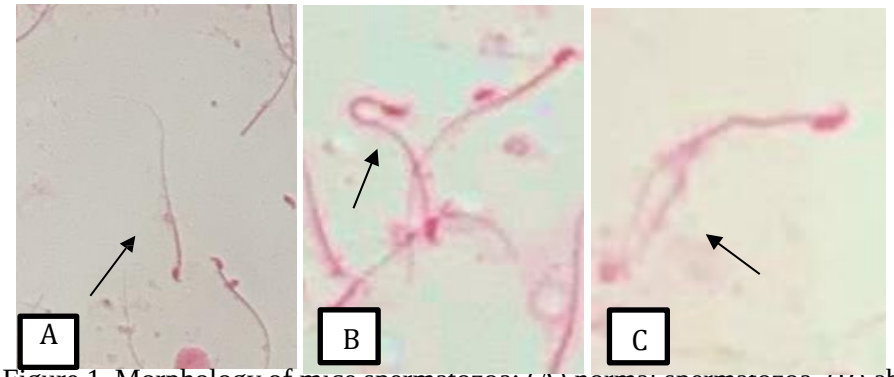

Figure 1. Morphology of mice spermatozoa; (A) normal spermatozoa, (B) abnormal middle piece, and (C) forked tail.

Observations for each treatment showed that the best group for increasing spermatozoa parameters was the P3 group. The data analysis is presented in Table 1. shows that the average number of spermatozoa of mice decreased in the aging model group (K-) which only amounted to 51.20 million/mL compared to the control group (K0) which amounted to 95.60 million/mL. This is due to the toxic effects of d-galactose. The number of spermatozoa increased to 51.20 million/mL (P1), 69.80 million/mL (P2), and the highest in (P3) to 82.20 million/mL. d-galactose stimulates the production of free radical compounds and interferes with carbohydrate metabolism, resulting in oxidative stress in the body. The increase in the levels of free radicals in the body causes cell apoptosis due to damage to the spermatozoa cell membrane, which can block the ion transport process required during the proliferation and growth stages of spermatogenic cells (Karim, 2011; Sukmaningsih, 2009). This situation interferes with protein degradation and cell proliferation, which causes DNA damage, undergoes apoptosis, and disrupts the cell cycle (Umbayev et al., 2020).

The presence of D-galactose will also cause interferes with the formation of testosterone, disruption of Sertoli cells to produce energy, disruption of mitosis, and proliferation of spermatogonium cells by decreasing the production of *Follicle Stimulating Hormone* (FSH) and *Luteinizing Hormone* (LH). Moreover, it will hinder hypothalamus function in blocking the anterior pituitary GnRh's function to secrete FSH and LH (Sari, Saebani, & Dhanardhono, 2018). Similar results were observed in terms of viability, with statistically significant variation. With an average score of 54.00%, the P3 group demonstrated the best treatment outcome, reaching the average value of the group with no treatment (K0). The average motility of spermatozoa increased in P3 by 65.00%. In addition, the best results for morphological observations were also found in P3, with an average of 63.00%. According to the results of this study, dead spermatozoa are red because the eosin dye has the ability to penetrate the plasma cell membrane due to damaged plasma membrane acrosome integrity, while viable spermatozoa are visible or colorless because the plasma membrane is still functioning effectively and cannot be penetrated by the eosin dye. The decrease in sperm parameters is due to the formation of AGEs by inducing D- galactose, which causes damage to the structure and function of mitochondria to increase and reduce the natural antioxidant system in the body (SOD), resulting in increased levels of ROS in the body. This situation could interfere with protein degradation and cell proliferation, leading to DNA damage and death, affecting the cell cycle (Umbayev et al., 2020). Oxidative stress interfere with the function of the epididymis and accessory glands, leading to a reduction in both the number and the quality of semen (Sutyarso, Annida, Kanedi, Busman, & Nurcahyani, 2018).

Furthermore, induction of d-galactose in the body stimulates the formation of ROS through the mechanism of oxidative metabolism of d-galactose and glycation end products (Tian et al., 2005). ROS aggression by D-galactose induction attacks the DNA, mitochondria, and plasma membrane of spermatozoa, which affects the movement of spermatozoa (Alahmar, 2019). This is due to a decrease in antioxidant levels caused by D-galactose, which inhibits the sperm activity of mice (David *et al*., 2022). *Reactive Oxygen Species* that attack the mitochondria damage the arrangement of DNA nitrogen bases and interfere with the formation of *Adenosine Triphosphate* (ATP). Mitochondria play an important role in the release of ATP during aerobic respiration, which is necessary for cell metabolism. In addition, the mitochondria regulate apoptosis and calcium homeostasis (Nakada et al. 2006). Increased sperm tail membrane integrity affects calcium influx and activates actin and myosin, which stimulates movement. Moreover, the presence of ROS induces a high amount of lipid peroxidation that causes damage and a decrease in the integrity of the spermatozoa cell membrane, which certainly reduces spermatozoa motility (Freitas, Vijayaraghavan, & Fardilha, 2017). Damage and decreased integrity of the spermatozoa cell membrane due to free radical attack can reduce sperm motility (Karim, 2011). In addition, Fitriani and Widya (2010) and Susmiarsih (2010) added that free radicals can also result in decreased mitochondrial ATP production, which functions in energy catabolism for spermatozoa tail movement. Spermatozoa motility requires energy derived from the mitochondria in the middle piece of spermatozoa cells because spermatozoa cell mitochondria contain ATP (Baszary, Kakisina, & Linda, 2021). Therefore, antioxidant compounds are needed as inhibitors of free radical attack. According to Christijanti, Utami, and Iswara (2010), antioxidant compounds are able to counteract and inhibit free radical attacks to maintain spermatozoa motility. This statement is also in line with Zulaikhah (2017), who reported that the presence of antioxidant compounds, both enzymatic

and non-enzymatic antioxidants that work together, can neutralize ROS and improve the performance of metabolic enzymes in the body.

The state of mice experiencing oxidative stress is caused by high levels of free radicals (ROS) that react with the surrounding cell molecules to obtain electrons so that they become stable. However, electrons from body cell molecules are converted into new free radicals and are reactive (Kunwar & Priyadarsini, 2011). High levels of ROS can also damage spermatozoa cell membranes, causing abnormalities in the morphology of mouse sperm. Oxidative stress in the body causes an oxidant attack on unsaturated fatty acids, causing lipid peroxidation (Lü, Lin, Yao, & Chen, 2010). According to Pamungkas (2013), if the percentage of abnormal spermatozoa is greater than 50%, it is said to be teratozoospermia caused by the accumulation of mitochondrial DNA (mtDNA) mutations, which are a source of energy for sperm. High levels of ROS can also cause damage to sperm cell membranes and DNA integration, leading to apoptosis (Sukmaningsih, 2009).

Lipid peroxidation of the spermatozoa cell membrane caused by reactive oxygen species (ROS) can damage the lipid matrix of the Sperm Cell membrane. This leads to damage to the sperm's central structure and acrosome, and influences the processes of capacitation and acrosome reaction, ultimately affecting fertility (Narayana, 2008). Consequently, ATP production decreases, spermatogenesis decreases, sperm morphology is abnormal, and sperm count decreases. Abnormal morphology of spermatozoa is caused by changes in Sertoli cells, which causes abnormalities in the proliferation of sperm cells in the epididymis (Quratul'ainy, 2006). The above situation will cause degeneration of spermatogenic cells characterized by vacuolization in the cytoplasm of seminiferous tubules and an indication of reduced Sertoli cell activity (Rumanta, Surjowo, & Sudarwati, 2001). Plasmasadow membranes of spermatozoa contain unsaturated fatty acids; therefore, ROS due to D-galactose induction causes lipid peroxidation to increase and damage the lipid structure of the plasma membrane (E, RI, & Purwantara, 2014). Antioxidant compounds capable of neutralizing these free radicals are required to compensate for the decrease in sperm count caused by free radicals.

Studies conducted by Dahlan and Tjokronegoro (2012) and Saleh and Agarwal (2002) showed that oxidative stress causes lipid peroxidation of spermatozoa plasma membranes, resulting in loss of motility, viability, capacitation ability, and damage to spermatozoa morphology. Kersen leaf extract was used in this study because kersen leaves contain many phytochemical compounds that act as antioxidants. The plasma membrane of spermatozoa contains unsaturated fatty acids; thus, ROS due to d-galactose induction causes lipid peroxidation to increase and damage the lipid structure of the plasma membrane (E et al., 2014). The active compounds possessed by kesen leaf are saponins, flavonoids, and tannins (Surjowardojo, Sarwiyono, & Ridhowi, 2014). In addition, polyphenolic compounds contained in kersen leaves have anti-glycation activity or inhibit glycosidation reactions by inhibiting RAGE signaling (Sadowska-Bartosz & Bartosz, 2015). Following the statement by Kuntorini, Fitriana, and Astuti (2013) that flavonoid compounds possessed by kersen leaves are natural antioxidant compounds and can be used as an antioxidant by kersen leaves is a natural antioxidant compound and has the effect of suppressing various oxidation reactions and can have the effect of suppressing various oxidation reactions and would act as a reducer of hydroxyl radicals, superoxide, and peroxyl radicals. Therefore, this study utilized kersen leaf extract as a source of antioxidants that are believed to suppress the attack of ROS in the body. Natural antioxidants are able to protect molecules and improve the quality of spermatozoa and increase the efficiency of male reproduction from cell damage due to ROS (George, Jiao, Bishop, & Lu, 2012; Hafez, 2010). According to Latif (2013), flavonoids act as primary antioxidants that can eliminate lipid peroxidation compounds by stopping hydrogen transfer in radical compounds. This is related to the research by Nishanthini, Ruba, and Mohan (2012), which showed that flavonoids and phenols can act as free radical neutralizers. Flavonoids are also able to increase the activity of antioxidant enzymes in the body, GSH, which can convert H_2O_2 and lipid peroxidation into H_2O. The GSH enzyme is located in the cytoplasm and functions on phospholipid membranes that undergo free radical oxidation (Setyaningsih, 2011). The effectiveness of Kersen leaf extract in increasing the number of spermatozoa is influenced by phytochemical compounds such as flavonoids, phenolic compounds, and antioxidants that actively increase testosterone, FSH, and LH hormones, thereby increasing the number of spermatozoa (Haron & Mohamed, 2016).

Kersen leaf extract contains ascorbic acid or Vitamin C, examples of water-soluble antioxidants that can decrease the production of free radicals and reduce lipid peroxidation in the testes (Aitken & Roman, 2008). Vitamin C acts as a scavenger, reducing free radicals in the plasma membrane, and protecting cells from ROS damage (Grosso et al., 2013).

Furthermore, flavonoids protect the plasma membrane from free radical attack, helping it suppress the entry of foreign molecules or substances that damage membrane integrity (Verstraeten, Oteiza, & Fraga, 2004). Polyphenolic chemicals that occur in Kersen leaf extract act as antioxidants, protecting DNA, lipids, and proteins from damage from free radicals (Dja'afara, Wantouw, & Tendean, 2015).

Giving antioxidant compounds such as kersen leaves will improve the physiological function of the anterior hypothalamus so that it can increase the work of Gonadotropin-Releasing Hormone (GnRH), which stimulates the anterior pituitary (Valli et al., 2014). The anterior pituitary will produce Luteinizing Hormone (LH), as well as Follicle Stimulating Hormone (FSH). LH stimulates the Leydig cells that produce testosterone (Pereira et al., 2017). The presence of FSH will induce Sertoli cells to produce *Androgen-Binding Protein* (ABP) which plays a role in the process of spermatogenesis (Berawi, Wahyudo, & Pratama, 2019).

4. Conclusion

Based on the results of the research that has been done, it is concluded that the induced Kersen leaf extract at a dose of 105 mg/kg body weight (P3) can increase the total, viability, motility, and morphology of mice spermatozoa after D-Galactose induction. However, this study had limitations in measuring malondialdehyde (MDA) levels as an indicator of increased free radicals. Therefore, further research is required on this topic.

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