ORIGINAL ARTICLE

Antiaging activities of *Muntingia calabura* Leaf Aqueous Extracts (MCE) on Testicular Histology, Spermatogenic Proliferation, and Testosterone Level of D-Galactose-Induced Mice Model

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ABSTRACT

INTRODUCTION: Male aging may disrupt spermatogenesis and induce intratesticular redox imbalance, leading to testicular damage. *Muntingia calabura* leaf extracts (MCE) have potent antioxidant activity, but *in vivo* reports in the male reproductive system aging are limited. This research aimed to determine the effects of MCE on testicular histology and testosterone levels in the D-galactose-induced aging mice model. **MATERIALS AND METHODS:** Plasma and testicular tissue obtained from 20 male *Mus musculus* strain Balb/C which were divided into 5 groups: normal; aging; aging+ *Muntingia calabura* leaf aqueous extracts (MCE) 35 mg/kg; aging+MCE 70 mg/kg; and aging+vitamin C 28 mg/kg. Treatments were scheduled orally for 4 weeks; aging condition was induced using oral 500 mg/kg D-galactose for 6 weeks before treatments. Plasma testosterone was determined using ELISA, seminiferous tubules' diameter, spermatogenesis score, and Leydig cell number were examined with routine staining, and the nuclear expression of Ki-67 was performed via immunohistochemistry. **RESULTS:** The MCE-treated aging group had higher seminiferous tubules' diameter, spermatogenesis score, Leydig cells' number, and percentage of cells expressing Ki-67 compared with the untreated group ($p < 0.005$), and the optimum dose was 70 mg/kg. However, testosterone levels of the MCE group did not significantly differ from those of others. **CONCLUSION:** Administration of MCE in D-galactose-induced aged mice improved seminiferous tubule diameter, spermatogenesis score, Leydig cells' count, and spermatogenic cell proliferation, but not testosterone level.

Keywords
Muntingia calabura, testosterone, aging, spermatogenesis, seminiferous tubule.

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INTRODUCTION

Aging is a normal mechanism, but occasionally prematurely happens due to DNA damage, which leads to cell regeneration failure.[1] This process is commonly induced by oxidative stress due to a disturbed balance of reactive oxygen species (ROS) formation and antioxidant system. Excessive ROS leads to lipid peroxide accumulation in the cell membrane and disrupts its functions and the declining cell viability.[2] ROS also reacts with polyunsaturated fats and forms malondialdehyde (MDA), a reactive aldehyde that is toxic for cells and tissues, decreasing tissues and organ functions and accelerating senescence.[3]

Senescence signs are visible in many organ systems, including the reproductive system.[4] Aged male reproductive system is characterized by a decrease in testicular volume, weight, and testicular density, which may disrupt the spermatogenesis.[5] While normal testicular tissue is essential for spermatogenesis, aging can induce testicular microstructural changes.[6] These changes include an increase in weight and thickness of tunica albuginea, thickening of the seminiferous tubule basement membrane, and also accumulation of collagen tissue and thickening of tunica propria of the testicles due to a reduction in testicular perfusion due to occlusion and

thickening of testicular blood vessels.[5,7,8] As the age progresses, intra-testicular antioxidant productions are declining.[6] This condition leads to an increased risk of testicular damage.[9,10]

Rodent models were frequently used in laboratory aging research. Naturally aged animals are the most ideal condition for mimicking natural aging in humans, but, due to problems concerning animals' lifespan, the chemical-induced aging models were also beneficial, for example, the D-galactose-induced aging models.[11] Inducing aging conditions using D-galactose was popular because it has many advantages such as being easy to apply, being low-cost, producing a high-survival animal, and having fewer side effects throughout the aging period.[12,13] In inducing male testicular aging models, D-galactose-induced male aging models were reported successfully to reproduce the aging mechanism.[14,15] Administering 50–500 mg/kg D-galactose daily for 6–8 weeks induced aging characteristics such as reduced testosterone and structural changes on the testis and spermatogenesis similar to those of aged control individuals aged 16 to 24 months.[16,17] Since one human year is nearly identical to nine mouse days when comparing their respective lifespans, therefore the animals in this study were equivalent to humans aged 52.60–78.90 years.[18]

Muntingia calabura (Jamaican cherry) is widely distributed in the tropics and subtropics, and is traditionally used as a gastric pain remedy. Leaves extracts of *M. calabura* have been reported to contain carbohydrates, protein, polyphenols, flavonoids, ascorbate acid, α-tocopherol, and chlorophylls.[19,20] *M. calabura* leaf extracts also have a potent antioxidant activity through 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and lipid peroxidase formation inhibitions.[21] This antioxidant activity has been confirmed in vitro and it is highly possible that this extract also has anti-aging properties. *M. calabura* leaf extracts *in vivo* exploration in Indonesia is quite narrow.[22,23] Based on this condition, in this study, we try to determine the potential usage of *M. calabura* leaves extracts as an antiaging agent, mainly in the male reproductive systems aging. In this research, we evaluated the antiaging properties of *M. calabura* leaf aqueous extract (MCE) on the male reproductive system, mainly on seminiferous tubules' diameter, spermatogenesis score, Leydig cells' number and expression of Ki-67 as a marker for the proliferation of spermatogenic cells and testosterone levels of D-galactose-induced aged mice.[24]

MATERIALS AND METHODS

Research Design

This study used a combination of pre-and post-test and the post-test only with a control design, conducted at the Integrated Research Laboratory, Islamic University of Indonesia in May-August 2021.

Experimental Intervention

This research is a continuation of previous research that studied the antiaging activities of MCE on D-galactose-induced *Mus musculus* Balb/c.[25] Research specimens were obtained from 20 male *Mus musculus* Balb/C, aged 12 weeks, and weighed 25 + 5 g. The sample size was determined using the Research equation.[26] Mice were grouped into normal control (I, received aquadest); aging control (II, received aquadest); aging group received MCE 35 mg/kg (III); aging group received MCE 70 mg/kg (IV); and aging group received vitamin C 28 mg/kg (V or positive control).[25] Aging conditions in Group II-V were induced with 500 mg/kg D-galactose orally (Sigma-Aldrich, USA) daily for 6 weeks.[9,17] After aging conditions were confirmed from elevated plasma MDA level, animals received scheduled treatment with 1 ml working solution (reconstituted with distilled water with a ratio of 1:1) orally for 4 weeks. The extract preparation used of *M. calabura* leaf collected from Sleman Region (GPS coordinate -7°.68751, 110°.41423), which was deposited and identified by Faculty of Mathematics and Natural Sciences, Islamic University of Indonesia (Register Number 02/UII/Jur.Far/det/I). After washing, air-dried and milled into powder; about 1,000 g of the powder was macerated with 5 l of distilled water for 24 hours and then filtered using filter paper. All filtrates from 3 × 24 hours were evaporated to obtain crude extract (Figure 1).

Retro-orbital blood samples were taken before and after treatment, while testicular tissues were taken after the animals' sacrifice via ketamine injection followed by

decapitation. The experimental protocols were approved by the Health and Medical Research Ethics Committee, Faculty of Medicine, Islamic University of Indonesia (Reg. Number 32/Ka.Kom.Et/70/KE/I).

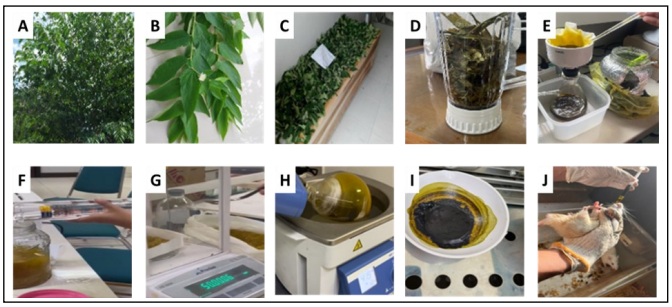

Figure 1. Preparation of *Muntingia calabura* leaves extract and administration to mice (A) *Muntingia calabura* tree; (B) Fresh leaves of *Muntingia calabura*; (C) Temperature and humidity controlled drying of collected leaves; (D) Pulverization of dried leaves; (E) Measurement of the ground material; (F) Maceration of leaves; (G) Filtration; (H) Evaporation; (I) Concentrated extract; (J) Administration of MCE via oral gavage

Sample Collection and Analyses

Blood samples were placed in containers, then centrifuged at 4,000 rpm (15 min), and retrieved plasma was stored at −20°C for further examination. Testosterone concentration was determined using ELISA (DRG® Testosterone, EIA-1559). Right testicles were placed in 10% buffer formalin and then processed into a paraffin block. The blocks were sectioned with a microtome of 5 µm thickness, deparaffinized, and stained for Hematoxylin-Eosin and analysed for spermatogenesis scores, Leydig's cells count (characterized by polygonal cells with eosinophilic cytoplasm and a large round nucleus with a prominent nucleolus), and seminiferous tubule's diameters.[27,28]

Table I: Johnsen's score on spermatogenesis[27]

Score	Criteria
Score 10	Complete spermatogenesis
Score 9	Moderately disturbed spermatogenesis, numerous late spermatids, disordered epithelium
Score 8	Less than 5 spermatozoa within a tubule, a small number of late spermatids
Score 7	No spermatozoa, no late spermatids, numerous early spermatids
Score 6	No spermatozoa, no late spermatids, a small number of early spermatids
Score 5	No spermatozoa or spermatids, plenty of spermatocytes
Score 4	No spermatozoa or spermatids, a small quantity of spermatocytes
Score 3	Spermatogonia only
Score 2	No spermatogenic cells, Sertoli cell only
Score 1	No seminiferous epithelium

Immunohistochemistry

After deparaffinized and processed in graded ethanol, the 5 µm tissue slices were incubated in H_2O_2 3% to eliminate nonspecific staining. After antigen retrieval process, the slides were incubated with Ki-67 primary antibody (Abcam, ab279653) at 4℃ overnight. The sections were incubated with secondary antibodies and then counterstained with Haematoxylin Meyer. The Ki-67 nuclei protein showed as brown-yellow particles and presented in percentage of KI-67 positive cells. All photomicrographs were taken with Olympus® CX23 (Olympus, Japan) assembled with Optilab® Viewer (Miconos, Indonesia). The quantification of all histology parameters was performed on 5 separate fields using Image Raster software (Miconos, Indonesia) by 2 observers blinded to the treatments.

Statistical Analysis

Data were reported in means and standard deviations and analysed with SPSS v.26.00 Statistics. Saphiro Wilk tests were conducted to assess data normality. For normally distributed data, the mean was compared using one way ANOVA and Tukey HSD for post hoc comparison. For non-normally distributed data, the mean was compared using Kruskal-Wallis followed by Mann-Whitney test. Wilcoxon signed-rank test was used to compare paired data (pre- and post- treatment). All tests were conducted in a 95% confidence interval.

RESULTS

Spermatogenesis Score and Morphological of Seminiferous Tubules

Examination of testicular tissue in the low magnification exhibited the normal control group had an intact seminiferous tubule with optimum epithelial thickness. The interstitial components of the normal control group occupy a small part of the field (Figure 2, Panel I, A). The aging control showed a dominant interstitial compartment with scanty distribution of seminiferous tubules and variable size of seminiferous tubules (Figure 2, Panel I, B). Most seminiferous tubules in the aging group had small diameters and thin epithelium layers. The aging group received 35 mg/kg MCE showed a dominant interstitial

compartment but thicker epithelium (Figure 2, Panel I, B). The aging group received 70 mg/kgBB MCE and vitamin C has a minimal interstitial compartment and thick epithelium (Figure 2, Panel I, D and E). The untreated aging group had the lowest diameter. While the aging group received MCE 70 mg/kg and vitamin C, showed a higher seminiferous tubule diameter, and did not significantly differ from the normal control. However, the MCE 35 mg/kg treated group still had lower diameters compared with the MCE 70 mg/kg and vitamin C group (Figure 3, A).

Higher magnification (400x) revealed thick epithelium with complete spermatogenesis which is made up of different spermatogenic cell phases, from spermatogonia, spermatocytes, spermatids, and spermatozoa (score: 10). The aging control group showed thin epithelium, cellular depletion, and minimal spermatogenesis (Figure 2, panel II B). Since the most common cells are immature sperm cells, the aging control group only reached a score of 6 in Johnsen's spermatogenesis scores (Figure 3, B). The treatment with MCE 35 mg/kg resulted in a similar histological appearance to the aging control group (Figure 2, Panel II C). However, the aging group received MCE 70 mg/kg showed thicker epithelium (Figure 2, Panel II D) and higher spermatogenesis score (score: 8-10, Figure 3B); vitamin C-treated group showed spermatogenesis score 9-10, but the score of both groups still lower compared with normal control. The treatment with MCE 70 mg/kg and vitamin C showed higher diameter and Johnsen's score compared with an untreated aging group ($p < 0.05$, Figure 3B).

Ki-67 Expression

The expression of Ki-67 in the seminiferous epithelium is related to actively proliferating and spermatogenic cells. The normal control group showed abundant cells expressing Ki-67 (Figure 2, Panel III A), mainly expressed in spermatogonia and spermatocytes, whereas the aging control showed a smaller number of cells (Figure 2, Panel III B). The aging group treated with MCE 70 mg/kg and vitamin C showed quite high levels of expression (Figure 2, Panel III D and E). The treatment with MCE 70 mg/kg and vitamin C showed a significant improvement in the percentage of Ki-67 positive cells, compared with an untreated aging group. Nevertheless, the aging group that received MCE 70/kg still had lower levels of Ki-67 expression than the normal control group ($p<0.05$, Figure 3C).

Leydig Cells' Count

The normal control group showed abundant interstitial cells of Leydig which produced testosterone. The aging group had the lowest number of Leydig cells, while the aging group received MCE 70 mg/kg and vitamin C showed an increased number of Leydig cells. The aging group received MCE 35 mg/kg showed an elevated number of Leydig cells, but did not significantly differ from the aging group. The number of Leydig cells in the aging group received vitamin C was similar to normal control, however, the MCE 70 mg/kg still had a lower number of Leydig cells ($p<0.05$, Figure 3D).

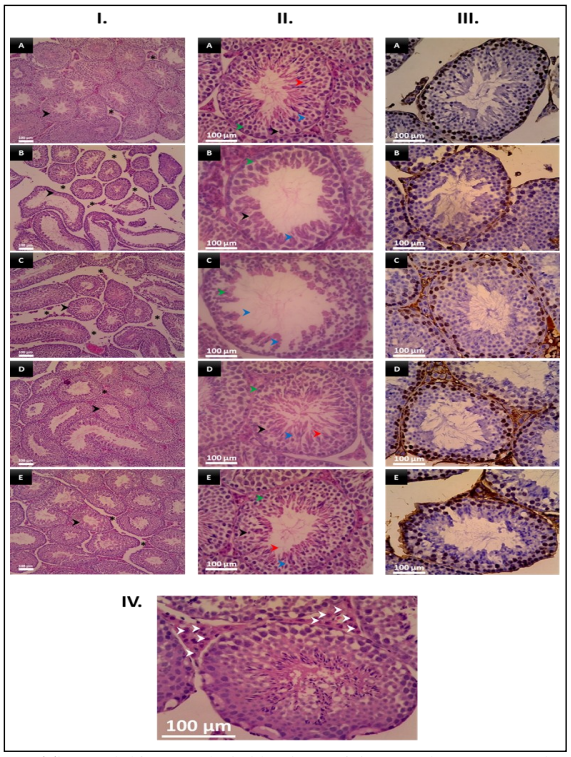

Figure 2. The seminiferous tubule histology of the experiment groups in various examinations. A: healthy control; B: aging control; C: Aging + MCE 35 mg/kg; D: Aging + MCE 70 mg/kg; E/ positive control: Aging + vitamin C 28 mg/kg. Panel I: The architecture of seminiferous tubules. The normal group showed thick epithelium and a small portion of interstitial space. While the aging group showed a reduced number of seminiferous tubules with small diameter and thin epithelium, but wide interstitial tissue. Bar indicated 100 µm in 100x magnification; Panel II: The seminiferous epithelium cells observation, spermatogonia (green arrow), spermatocytes (black arrow), spermatozoa (red arrow), and spermatids (blue arrow), bar indicated 100 µm in 400X magnification; Panel III: Nuclear expression of Ki-67 (brown-coloured) with Ki-67 immunohistochemistry staining. The Ki-67 expression was higher in the healthy control compared to the aging control group. Bar indicated 100 µm in, 100X magnification; Panel IV: Interstitial cell of Leydig (arrowheads) in the testicular interstitial tissue. Bar indicated 100 µm in, 400X magnification. MCE = *Muntingia calabura* leaves aqueous extract.

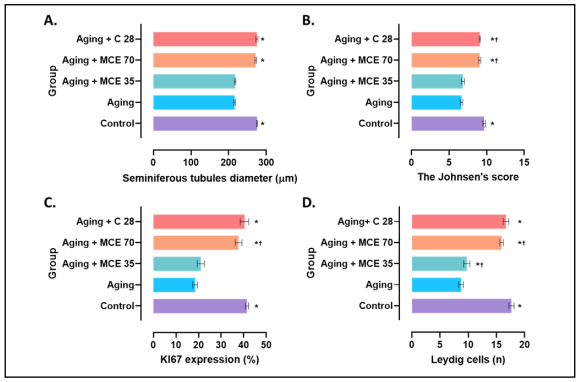

Figure 3: Seminiferous tubule parameters; (A) Comparison of tubules' diameter. The aging group and aging + MCE 35, exhibit the smallest seminiferous diameter, compared to the rest of the experimental groups; (B) Johnsen's spermatogenesis scores. The control group has the highest Johnsen's score. (C) Comparison of Ki-67 positive cell percentages. The control group shows the highest Ki-67 positive cell percentages among the experimental groups, followed by the positive control group. (D) The number of Leydig cells for each experimental group. Signifier symbol: *p < 0.05 compared with the aging group, †p < 0.05 compared with a control group (Analysis of Variance and post hoc Tukey Honestly Significant Difference analyses).

Testosterone Level

Table II presented the mean levels of testosterone. Overall, the increase in testosterone level is 0.87 ± 4.26 ng/mL. In the control group, pre-treatment testosterone levels ranged from 0.17-0.27 ng/mL with a mean of 0.24 ± 0.05 ng/mL. At the end of the research period, we noted an insignificant increase of about 3.21 ± 5.87 ng/mL compared with the pre-test testosterone level.

Pre-treatment testosterone of aging control was 3.37 ± 5.91 ng/mL. Post-treatment measurement reveals a decrease in testosterone level, about 2.58 ± 5.22 ng/mL, which was the lowest testosterone level among groups. The mean pre-treatment testosterone levels of the aged group received 35 mg/kg of MCE were 0.43 ± 0.02 ng/mL. During treatment periods, the testosterone levels rose by about 0.54 ± 0.78 ng/mL, and at the end of treatment periods, post-treatment testosterone levels for this group were 0.96 ± 0.80 ng/mL. Before being treated with MCE 70 mg/kg, the mean value of testosterone levels of group IV was 0.52 ± 0.04 ng/mL. Post-treatment testosterone levels were 3.31 ± 4.89 ng/mL, which means there was an increase in testosterone level, for about 2.79 ± 4.86 ng/mL. Pre-treatment testosterone levels of the aged group treated with 28 mg/kg of vitamin C were 0.51 ± 0.11 ng/mL. After vitamin C treatment, the testosterone levels decreased to 0.90 ± 0.65 ng/mL, reducing 0.39 ± 0.62 ng/mL of testosterone levels. Kruskall-Wallis's analysis revealed a significant difference in pre-treatment testosterone levels (p-value= 0. 025), but no significant difference in testosterone escalations (p-value= 0. 734) and post-treatment testosterone levels (p-value= 0. 282). In post-hoc analysis, only Group IV that has a significantly different testosterone level, compared with the control group (p-value= 0. 282).

Table II: Testosterone level of *Mus musculus* Balb/c

Testosterone level			**Testosterone level range**		**Mean ± SD (ng/mL)**	
Pre-treatment (group I-V)			0.17-12.23		1.01 ± 2.64	
Post-treatment (group I-V)			0.36-12.29		1.88 ± 3.33	
Testosterone escalation (post-pre)			-10.40-12.02		0.87 ± 4.26	
	Pre-treatment testosterone levels (ng/mL) *		Post-treatment testosterone levels (ng/mL)		Testosterone escalations (ng/mL)	
	Range	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD
Group I	0.17 - 0.27	0.24 ± 0.05	0.42 - 12.29	3.46 ± 5.90	0.16 - 12.02	3.21 ± 5.87
Group II	0.28 - 12.23	3.37 ± 5.91	0.38 - 1.83	0.79 ± 0.69	-10.40 - 0.24	-2.58 ± 5.22
Group III	0.41 - 0.45	0.43 ± 0.02	0.49 - 2.16	0.96 ± 0.80	0.07- 1.71	0.54 ± 0.78
Group IV	0.49 - 0.57	0.52 ± 0.04**	0.52 - 10.63	3.31 ± 4.89	0.02 - 10.06	2.79 ± 4.86
Group V	0.42 - 0.66	0.51 ± 0.11	0.36 - 1.82	0.90 ± 0.65	-0.06 - 1.31	0.39 ± 0.62

*p< 0.05 on Kruskal Wallis test; **p< 0.05 on Mann-Whitney post hoc, compared to the control group (Group I). I: healthy control, II: aging control, III; Aging + MCE 35 mg/kg, IV: Aging + MCE 70 mg/kg, V: Aging + vitamin C 28 mg/kg. MCE: *Muntingia calabura* extract. SD: standard deviation

DISCUSSION

Spermatogenesis is a complex process involving the mitotic division of the spermatogonia, to the formation of mature and motile sperm cells. Optimal spermatogenesis also required many factors, including the role of the interstitial compartment of the testis, mainly the testicular vascularization and the interstitial cell of Leydig.[29] However, the spermatogenesis process was disrupted in aged males. Sperm cell death rises with age, and spermatogenesis impairment is primarily shown in spermatocyte meiosis and spermatogonia mitosis, which leads to fewer sperm cells and sperm distortion.[5, 6, 30]

In this study, we use D-galactose to induce testicular aging in mice. Oral gavage of D-galactose was successful in elevating plasma MDA concentration compared with the normal group, and 4 weeks of MCE treatment and vitamin C significantly reduced plasma MDA levels, also, the testosterone level of the aging control group was decreased after six weeks of D-galactose induction.[17,25]

Chronic administration of D-galactose can induce aging conditions in the male reproductive system.[9,17]

D-galactose can cause a decrease in testicular weight and volume, poor quality of sperm analysis (including sperm count, motility, and morphology of sperm cells), lower testosterone levels, also a decrease in spermatid and spermatozoa gene expression markers, a downregulation of testosterone synthesis, which progresses to impaired spermatogenesis.[9,31] They reported that 6-8 weeks administration of oral low-dose-D-galactose accelerates aging conditions in the male reproductive system, and the condition resulting from this method of induction resembles natural aging with similar changes in reduced sperm count, reduced ratio of testicular weight/body weight with parenteral administration. Animals that received oral administration of D-galactose also had a reduced activity of superoxide dismutase and increased level of testicular lipid peroxidation.[12] The reduced testicular mass might be caused by testicular tubular size reduction, spermatogenesis failure, and reduction of Leydig cell activity.[9] Animals that received D-galactose had disturbed seminiferous epithelial structure and decreased number of spermatogenic cells; enlarged congested blood vessels and cellular exudates in the interstitial compartments.[32] Our study also established D-galactose as a method for inducing male reproductive system aging based on the decreased level of testosterone, decreased diameter of seminiferous tubule, decreased spermatogenesis score and percentage of spermatogenic cell expressed KI-67, which is widely known as a marker for cellular proliferation.

We reported that administration of MCE 70 mg/kg improved the testicular morphology of the D-galactose aging mice model and improved the rate of KI-67 positive cells. However, although groups receiving MCE 70 mg also exhibit a higher number of interstitial cells of Leydig, the testosterone level of this group did not significantly differ from the aging control group. These results might reveal the potency of the antiaging properties of MCE on testicular aging conditions induced by D-galactose.

Many research reports that all parts of *M. calabura* comprise active compounds including proteins, carbohydrates, polyphenols, flavonoids, ascorbic acid, α-tocopherol, and chlorophyll. These chemicals are recognized for their antioxidant properties and possess the ability to inhibit oxidative stress.[19] *M. calabura* contains polyphenol compounds such as gallocatechin, epigallocatechin, catechin, flavonols, naringenin, quercetin, and gallic acid which are reported to have antioxidant capacity.[33] *M. calabura* leaf extracts are reported to have antioxidant activity which was examined on reducing the level of DPPH, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radicals, and also iron reduction.[34] *M. calabura* leaf extracts are known to have the capability to inhibit DPPH and also have intracellular antioxidant activity.[35,36] The antioxidant capacities of the MCE have corresponded to total phenols.[34] The antioxidant capacity of *M. calabura* is relevant to the high number of flavonoids and phenolic constituents.[37] Total phenols and flavonoids analysis using colorimetric methods reported that total phenols and flavonoids detected in the MCE were 2.19 + 0.12 mgGAE/g and 2.43 + 0.24 mgQE/g respectively.[38] Also, the presence of myrcene, thymol, α-terpinol, linalool, geraniol, nerol, citronellol, eugenol, α-lonone, β-sitosterol, α-amyrin, lupelol, α-tocopherol, and β-carotene were reported in aqueous leaf extract.[20] Studies on the antiaging potency of MCE in the male reproductive system are scarce. *M. calabura* leaf extract might be slowing down the aging process by inhibiting free radicals including Advance Glycation End products (AGEs) and ROS.[39] Also, the polyphenolic compounds of MCE have antiglycation activity through inhibition of receptors for AGEs signaling, and inhibiting glycosylation.[40]

In vitro research reported that *M. calabura* fruit is a promising candidate for an antiaging agent for skin rejuvenation. Besides having antiradical activity, the *M. calabura* fruit lyophilisate was also reported to have anti-aging activity on the inhibition of elastase with IC50 of 21.67 and collagenase and with IC50 of 180.61 μg/mL.[36] Both enzymes were known to be responsible for collagen and elastic fiber degeneration in aging. Therefore, the inhibitions of those enzyme activities were subjects for anti-aging research.[41] The lyophilisate of the *M. calabura* fruit also preserves and augments fibroblast's viability in vitro and also increases the amount of procollagen-1-propeptide N-terminal (P1NP), a marker for

fibrogenesis.[36,42]

Nonetheless, the MCE administration did not result in an improvement and elevation of serum testosterone levels. The fluctuation of testosterone levels creates conditions in this research. The mean testosterone levels before (1.01 ± 2.64 ng/mL) and after treatment (1.89 ± 3.33 ng/mL) are within the normal limit. However, if we examine the average value of testosterone levels per group, either before or after treatment, most of the groups had lower testosterone levels than normal mice testosterone levels, which range from 1.5-2.0 ng/mL.[43] There are variations in testosterone levels of normal male mice, for instance, normal male mice's testosterone levels range between 2.8-9.5 ng/mL, and the mean testosterone level is 6.6 + 1.9 ng/mL or serum testosterone levels range in the lower level (44.80 ng/dl or 0.45 ng/mL).[44] Previous study reported a significant difference in *Mus domesticus* testosterone levels at the beginning of the study (0.24-37.7 ng/mL with a mean of 5.62 ng/mL) and when the study was completed (0.19-60.1 ng/mL with a mean of 11.2 ng/mL).[45] Repeated measurements of individual mice testosterone levels, indicating a significant fluctuation, range from 1.1-32 ng/mL with an increase of 2-5-fold and the coefficient of variation reaches 82% in everyone.[46,47] In addition, there were differences in the range and mean of testosterone levels as measured by different methods and tools, therefore the normal testosterone levels of test animals were likely to be strongly influenced by measurement methods and tools or materials used.[48]

We suspect that the testosterone levels in the stored sample used in this study were changed due to the storage process. There is a change in testosterone levels by 23.9% in blood samples which are stored at 22°C for a day.[49] In contrast, other research reported duration of storage time did not affect the testosterone levels of the measured samples, valid measurements are still feasible.[50] This phenomenon should be carefully examined in the next research to achieve conclusive results.

CONCLUSIONS

Administration of *M. calabura* leaf extract improved the testicular histology (diameter of the seminiferous tubule, spermatogenesis score, Leydig cells' count, and spermatogenic cell proliferation) in D-galactose-induced aging mice model but showed no significant effect on testosterone levels.

CONFLICT OF INTEREST

None to declare.

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