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The influence of ethanol extract of dayak onion (*Eleutherine palmifolia*) on the number of Leydig cells and seminiferous tubules diameter in mice (*Mus musculus*) exposed to monosodium glutamate

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Abstract

Objective: This study aimed to investigate the preventive effect of ethanol extract from dayak onion (*Eleutherine palmifolia*) on the number of Leydig cells and the diameter of seminiferous tubules in mice (*Mus musculus*) exposed to monosodium glutamate (MSG).

Method: A total of 25 mice were divided into 5 groups. Group C- received only 0.5% CMC-Na. Group C+ was given 0.5% CMC-Na and 4 mg/gBW/day of MSG. Groups T1, T2, and T3 were administered dayak onion (*Eleutherine palmifolia*) ethanol extract at doses of 30, 60, and 120 mg/kgBW/day, respectively, along with 4 mg/gBW/day of MSG. All treatments were administered orally for 52 days.

Result: The results indicated that MSG exposure resulted in a significant decrease in the number of Leydig cells and the diameter of seminiferous tubules in the C+ group compared to the C- group ($p < 0.05$). Administration of dayak onion (*Eleutherine palmifolia*) ethanol extract at doses of 30 (T1), 60 (T2), and 120 mg (T3) per kg BW showed similar results ($p>0.05$) to those of the C- group.

Conclusion: In conclusion, the administration of dayak onion (*Eleutherine palmifolia*) ethanol extract mitigates the harmful effects of MSG on the number of Leydig cells and seminiferous tubules diameter in mice (*Mus musculus*) as a model. This research shows that Dayak onion (*Eleutherine palmifolia*) plays a role in reproductive health care.

Keywords: Dayak Onion; MSG; Leydig Cells; Seminiferous Tubules Diameter; Reproductive Health Care

1. Introduction

Monosodium glutamate (MSG) is one of the commonly used food enhancers, especially in commercial foods [1]. The use of MSG has been prevalent for over 100 years and has seen an increase in recent times. MSG consumption reached 9.62 g/capita/day in 2011, compared to just 1.53 g/capita/day in 1998 [2]. Prolonged consumption of MSG can lead to its accumulation in the body, causing various effects on tissues or organs, including reproductive system damage and infertility [3]. Infertility caused by chronic MSG consumption is due to the formation of reactive oxygen species (ROS) in the testes [4]. Excessive ROS production leads to oxidative stress and lipid membrane peroxidation, marked by increased malondialdehyde (MDA) levels and decreased glutathione (GSH) levels [5].

Consistent with the research by Luqman et al., who found that administration of 4 mg/gBW MSG to mice (*Mus musculus*) led to increased MDA levels and Leydig cell necrosis [6]. The reduction in the number of Leydig cells and seminiferous

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tubules' diameter occurs due to hormonal disruption caused by free radical formation. Excess glutamic acid increases the expression of glutamate receptors mGluR, iGluR, and NMDAR, leading to the release of Ca2+ into cells and tissues mediated by nitric oxide (NO) production, resulting in mitochondrial dysfunction and superoxide production [7, 8]. The reaction between NO and superoxide can lead to lipid peroxidation, direct DNA damage, and protein dysfunction [7]. mGluR receptors also activate the phospholipase C (PLC) pathway through G protein activity, producing inositol trisphosphate (IP3) and increasing intracellular Ca^{2+}, leading to NMDAR receptor activation and intracellular Ca^{2+} increase [7]. This mechanism increases ROS production and causes excitotoxicity in neurons. Neuronal oxidative stress leads to damage in the hypothalamus-pituitary-testis axis, affecting follicle-stimulating hormone (FSH) and luteinizing hormone (LH) [9]. Research by Kalsum et al. showed that oral administration of 4 mg/gBW MSG for 30 days in male mice led to a reduction in seminiferous tubules' diameter and the number of spermatogenic cells due to oxidative stress [10].

Efforts to reduce the oxidative stress caused by free radicals can be achieved through antioxidant administration. Antioxidants can neutralize unstable free radicals by donating electrons [11]. Plants containing antioxidants can serve as natural antioxidants to counteract free radicals. One such plant is dayak onion (*Eleutherine palmifolia*). Dayak onion contains antioxidant compounds such as flavonoids, tannins, and phenols [12]. Chemical compounds found in dayak onion include 15 compounds, including flavonoid derivatives and naphthoquinones [13]. High antioxidant activity is attributed to flavonoid compounds [14], indicating that dayak onion has a high antioxidant activity against free radicals. Another study by Gayatri et al. found that administering ethanol extract of dayak onion at doses of 30, 60, and 120 mg/kg BW to male mice induced with lead acetate for 35 days increased the diameter of seminiferous tubules in mice [15].

This research was conducted to determine the effect of administering ethanol extract of dayak onion at various doses to maintain the number of Leydig cells and the diameter of seminiferous tubules in mice exposed to MSG. Ethanol extract of dayak onion is expected to prevent or reduce the toxicity caused by monosodium glutamate.

2. Materials and Methods

2.1. Research Design

This research obtained ethical approval for the use of experimental animals from FKH UNAIR with certificate number 1.KEH.081.07.2022. The research design used in this study was a laboratory experimental design using a Completely Randomized Design (CRD). This research used 25 male mice (*Mus musculus*) aged 11 weeks with an average body weight of 20 g, obtained from the Pusat Veterinaria Farma (Pusvetma) in Surabaya, Indonesia. The testicular samples were collected from a group of mice subjected to treatments for 52 days. There were five treatment groups, each consisting of five mice. The ethanol extract of dayak onion was prepared in the Division of Clinical Pathology, Division of Basic Veterinary Medicine, Faculty of Veterinary Medicine. The experimental animals were housed in the Animal Husbandry Unit of the Faculty of Veterinary Medicine. Histopathological preparations of the testes and interpretation of research results were conducted in the Division of Veterinary Pathology, Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia.

2.2. Materials

The materials used in this research included 25 male mice (*Mus musculus*) aged 11 weeks with an average body weight of 20 g, BR-1 pellets, zeolite sand, distilled water, dried dayak onion, 96% ethanol solvent, monosodium glutamate (MSG) with production code N2 240923 produced in China, 0.5% CMC-Na, 10% neutral formalin buffer, alcohol solutions with concentrations of 70%, 80%, 90%, 95%, and absolute alcohol, xylene solution, liquid paraffin, and Hematoxylin Eosin stain.

Equipment and instruments used in the research included plastic containers measuring 45 x 36 x 15 cm, wire mesh, special drinking bottles for mice with a volume of 80 ml, a digital scale brand Camry with a precision of 0.1 g, sterilized glass bottles, probe instruments, 1 cc tuberculin syringes brand onemed. Minor surgical instruments included hand gloves, masks, tweezers, scalpels, scissors, and organ pot bottles. Equipment for histopathological preparations included object glass, cover glass, automatic tissue processor, water bath, hot plate, microtome, blade, optic lab camera, and microscope (Olympus® CX-21).

2.3. Methods

The first step upon arrival of the mice was to prepare cages measuring 45 x 36 x 15 cm with a zeolite sand floor. Before the research was conducted, the mice were acclimatized for 7 days in the cages with appropriate temperature, humidity,

lighting, and sanitation conditions. They were provided with pellet feed and distilled water ad libitum. The treatment could begin on the eighth day after the mice's acclimatization.

2.3.1. Dayak Onion Extraction Process

This study used dried dayak onion bulbs exposed to sunlight. The procedure for making ethanol extract of dayak onion involved grinding 300 g of dried dayak onion into powder. The powdered dayak onion was macerated in a 1-liter solution of 96% ethanol for 3x24 hours, followed by filtration using flannel cloth. The filtrate was subjected to two more maceration cycles with the same treatment until it became clear. Then, the filtrate was evaporated in a rotary evaporator at a temperature of 40-50°C with a speed of 40 rpm to obtain a concentrated extract [16]. This concentrated extract was transferred into sterilized glass bottles and stored in a refrigerator.

2.3.2. Dose Determination

The determination of dayak onion extract doses was based on the research by Gayatri et al. [15], which showed that ethanol extract of dayak onion at doses of 30, 60, and 120 mg/kgBW in male mice induced with lead acetate at 0.75 mg/kgBW/day orally for 35 days increased the diameter of seminiferous tubules. The dose of monosodium glutamate (MSG) used was 4 mg/gBW, based on the research by Kalsum et al., which found that oral administration of MSG to mice for 30 days reduced the diameter of seminiferous tubules [10], and Luqman et al., who reported that MSG administration to mice for 52 days increased Leydig cell necrosis and MDA levels [6].

2.3.3. Experimental Treatment

This study consisted of five groups: Group (C-): received only 0.5% CMC-Na. Group (C+): orally administered 0.5% CMC-Na and 4 mg/g BW MSG. Group (T1): orally administered ethanol extract of dayak onion at a dose of 30 mg/kgBW/day and 4 mg/g BW MSG. Group (T2): orally administered ethanol extract of dayak onion at a dose of 60 mg/kgBW/day and 4 mg/g BW MSG. Group (T3): orally administered ethanol extract of dayak onion at a dose of 120 mg/kgBW/day and 4 mg/g BW MSG. All groups received the treatments orally in a volume of 0.2 ml according to their respective groups from day 1 to day 52. On day 53, surgery was performed on all mice by cervical dislocation, followed by abdominal incision to collect the testes, which were then placed in a 10% neutral formalin buffer solution for histopathological preparation with Hematoxylin Eosin staining.

2.3.4. Data Collection

The number of Leydig cells in the testes was counted in five interstitial tissue fields in one preparation, and the averages were calculated. Observation was carried out using a microscope at 400x magnification with the assistance of image raster [17]. The diameter of seminiferous tubules was measured four times by drawing lines on the shortest and longest distances connecting the basement membrane of the seminiferous tubules in five fields of view in one preparation, and the average was calculated. Observation was conducted using a microscope at 100x magnification with the assistance of image raster, and the measurement results were expressed in micrometers (µm) [18].

2.3.5. Data Analysis

Data analysis obtained from the calculation of the number of Leydig cells and the diameter of seminiferous tubules was analyzed using a one-way ANOVA parametric test. If there were significant differences among the research groups ($p<0.05$), then Duncan's multiple range test was performed to determine differences between each treatment group. Data analysis was conducted using the Statistical Product and Service Solution (SPSS) version 25.0.

3. Results

The results of the study on the influence of ethanol extract of dayak onion (*Eleutherine palmifolia*) on the number of Leydig cells and the diameter of seminiferous tubules in mice (*Mus musculus*) exposed to monosodium glutamate showed significant differences ($p<0.05$) among the groups.

3.1. Number of Leydig Cells

The results of data analysis using one-way ANOVA showed significant differences ($p <0.05$) among the C (-), C (+), T1, T2, and T3 groups. The results of Duncan's multiple range test indicated that the C (-) group was significantly different from the C (+), T1, T2, and T3 groups. The C (+) group showed significant differences from the C (-), T1, T2, and T3 groups. The T1 group showed significant differences from the C (-), C (+), and T3 groups, but not significantly different from the P2 group. The P2 group showed significant differences from the C (-), C (+), and T3 groups, but not significantly different from the T1 group. The T3 group showed significant differences from the C (-), C (+), T1, and T2 groups. The

histopathological representation of Leydig cells in the negative control group C (-), positive control C (+), T1, T2, and T3 can be seen in Figure 1.

Table 1 Mean number of Leydig cells and diameter of seminiferous tubules in the testes of mice (*Mus musculus*) treated with ethanol extract of dayak onion (*Eleutherine palmifolia*) at various doses with monosodium glutamate exposure.

Treatment	Number of Leydig Cells (Mean ± SD)	Seminiferous Tubules Diameter (µm) (Mean ± SD)
C (-)	$29.88^{a} \pm 1.72$	$185.25^{a} \pm 3.62$
C (+)	$15.80^{d} \pm 1.65$	$148.62^{e} \pm 2.18$
T1	$20.36^{c} \pm 0.82$	$155.13^{d} \pm 3.11$
T2	$22.16^{c} \pm 1.20$	$165.03^{c} \pm 3.58$
T3	$26.60^{b} \pm 1.24$	$177.99^{b} \pm 1.50$

Note: Different superscripts in the same column indicate significant differences ($p < 0.05$). C (-): CMC Na 0.5%. C (+): MSG 4 mg/gBW and CMC Na 0.5%. T1: Ethanol extract of dayak onion 30 mg/kgBW and MSG 4 mg/gBW. T2: Ethanol extract of dayak onion 60 mg/kgBW and MSG 4 mg/gBW. T3: Ethanol extract of dayak onion 120 mg/kgBW and MSG 4 mg/gBW.

Figure 1 shows the number of Leydig cells in the C (-) group has the highest amount among the treatment groups. The C (+) group experienced a significant decrease in the number of Leydig cells, and the interstitial tissue appeared to undergo atrophy, resulting in irregular spacing between seminiferous tubules. The T1, T2, and T3 treatment groups had more Leydig cells than the C (+) group. The T3 treatment group, with a dosage of 120 mg/kgBW/day of dayak onion ethanol extract, showed the most effective dose in maintaining the number of Leydig cells compared to other treatment groups.

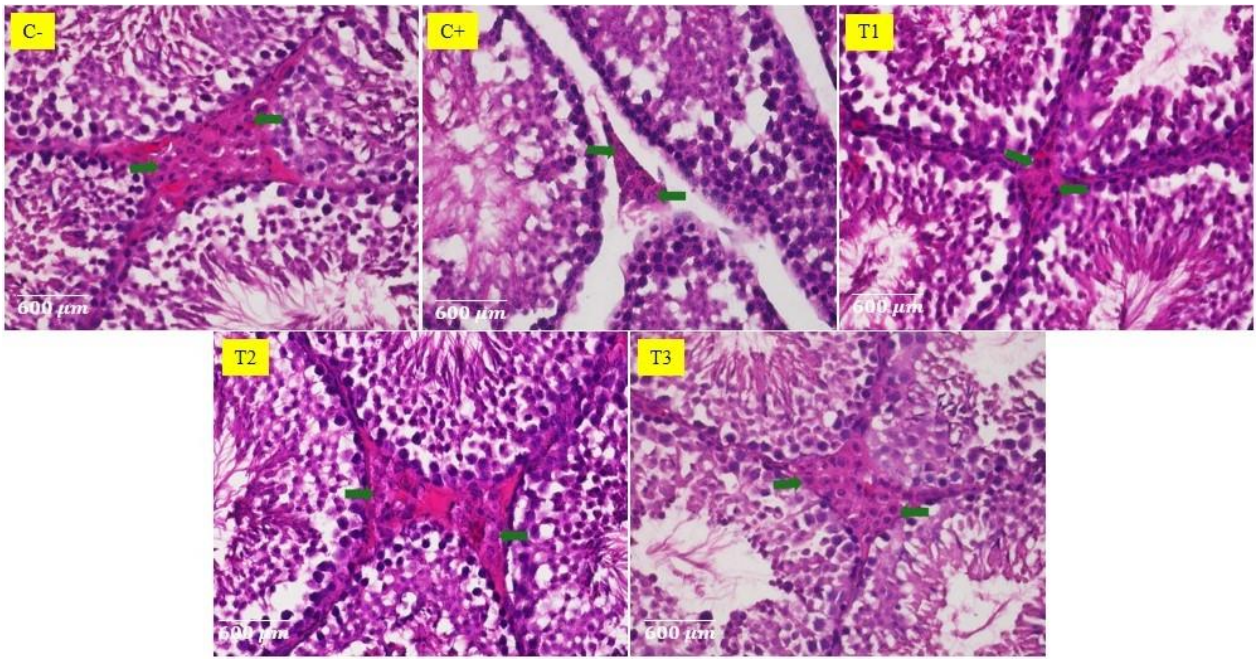

Figure 1 Histopathological representation of Leydig cells at 400x magnification with HE staining in the C (-), C (+), T1, T2, and T3 groups. Green arrows (➡) indicate Leydig cells. The number of Leydig cells (green arrows) is highest in C (-) and decreases significantly in C (+). Subsequently, there is an improvement in T1, T2, and T3, with the highest number in T3. C (+): MSG 4 mg/gBW and CMC Na 0.5%. T1: Ethanol extract of dayak onion 30 mg/kgBW and MSG 4 mg/gBW. T2: Ethanol extract of dayak onion 60 mg/kgBW and MSG 4 mg/gBW. T3: Ethanol extract of dayak onion 120 mg/kgBW and MSG 4 mg/gBW.

3.2. Seminiferous Tubules Diameter

The results of data analysis using one-way ANOVA showed significant differences ($p < 0.05$) among the C (-), C (+), T1, T2, and T3 groups. The results of Duncan's multiple range test indicated significant differences among the C (-), C (+), T1, T2, and T3 groups.

The histopathological representation of seminiferous tubule diameter in the negative control group C (-), positive control C (+), T1, T2, and T3 can be seen in Figure 2. Figure 2 shows the seminiferous tubule structure in the C (-) group has the widest diameter among the treatment groups. The C (+) group experienced a decrease in the diameter of seminiferous tubules compared to other treatment groups, resulting in irregularly spaced tubules. The T1, T2, and T3 treatment groups showed an improvement in the diameter of seminiferous tubules compared to the C (+) group. The T3 treatment group, with a dosage of 120 mg/kgBW/day of dayak onion ethanol extract, demonstrated the most effective dose in maintaining the diameter of seminiferous tubules compared to other treatment groups.

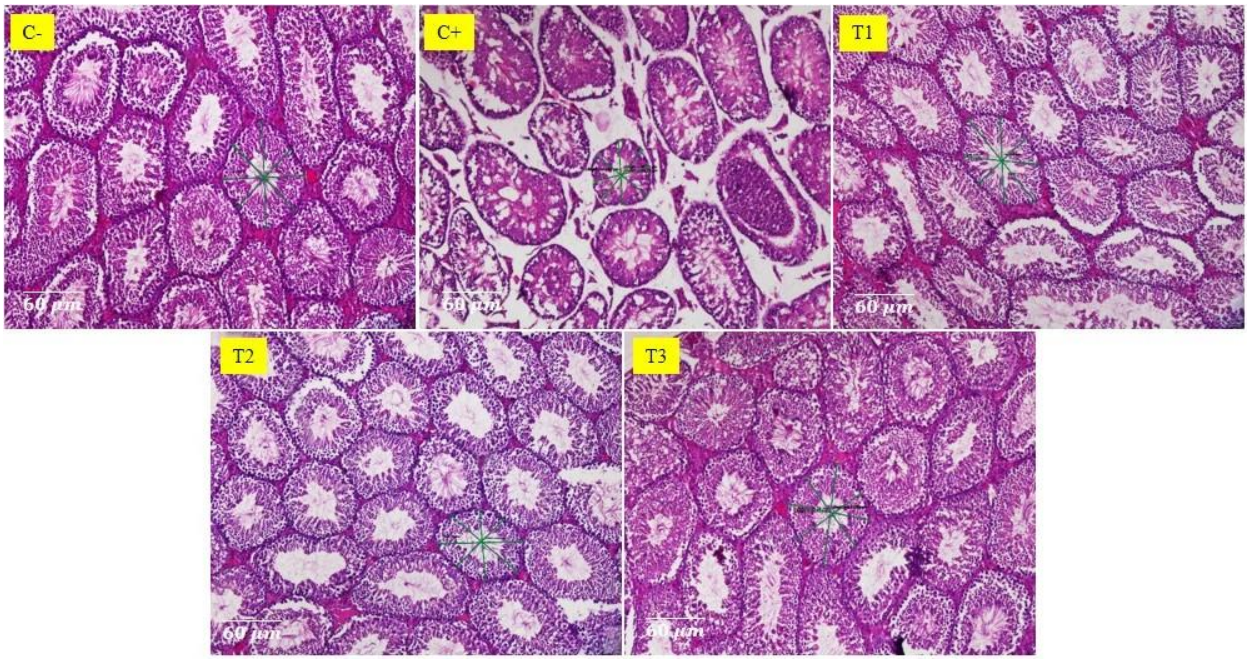

Figure 2 Histopathological representation of seminiferous tubules at 100x magnification with HE staining in the C (-), C (+), T1, T2, and T3 groups. Green arrows (➡) indicate the diameter of seminiferous tubules. The diameter of seminiferous tubules (green arrows) is widest in C (-) and decreases in C (+). Subsequently, there is an improvement in T1, T2, and T3, with the widest diameter in T3. C (+): MSG 4 mg/gBW and CMC Na 0.5%. T1: Ethanol extract of dayak onion 30 mg/kgBW and MSG 4 mg/gBW. T2: Ethanol extract of dayak onion 60 mg/kgBW and MSG 4 mg/gBW. T3: Ethanol extract of dayak onion 120 mg/kgBW and MSG 4 mg/gBW.

4. Discussion

Several factors and mechanisms can play a role in the cell death process in the testes, including increased oxidative stress, cell damage due to free radicals, mitochondrial dysfunction, and glutamate excitotoxicity [19]. Reactive oxygen species (ROS) in the body, such as hydroxyl radicals (OH•), superoxide anions (O_2•-), and peroxyl radicals (H_2O_2), can be formed due to excessive monosodium glutamate (MSG) exposure in the body [20]. Excessive free radicals induce oxidative stress [21]. Prolonged oxidative stress can cause cell damage in three ways: lipid membrane peroxidation leading to cell membrane damage, DNA damage resulting in DNA mutations and cell death, and oxidized protein modifications [22].

Leydig cells are located in the interstitial tissue and function as testosterone producers in response to interstitial cell stimulating hormone (ICSH) [23]. Excessive glutamic acid leads to oxidative stress in the nervous system, which reduces Leydig cell function and spermatogenesis processes. Increased glutamate levels cause the entry of Ca^{2+}, mediated by nitric oxide (NO) production, resulting in mitochondrial dysfunction and the generation of superoxide [8]. The reaction between NO and superoxide can produce peroxynitrite, which can lead to lipid peroxidation, direct DNA damage, and protein dysfunction [7].

Based on the research results, the positive control group C (+) exposed to 4 mg/gBW/day of MSG showed a significant decrease in the number of Leydig cells compared to the negative control group C (-) which only received 0.2 ml/day of CMC Na 0.5%. This is in line with Luqman et al.'s study, which showed an increase in Leydig cell necrosis and MDA levels in male mice exposed to MSG for 52 days [6]. This decrease is caused by Leydig cell damage due to increased oxidative stress from glutamic acid accumulation in the testes. Excessive glutamic acid increases the expression of metabotropic glutamic receptor (mGluR), ionotropic glutamic receptor (iGluR), and N-methyl-D-aspartate receptor (NMDAR) [7]. All three receptors will activate the phospholipase C (PLC) pathway through G protein activity, which produces inositol trisphosphate (IP3) and increases Ca^{2+}, causing oxidative stress in cells and tissues [8]. NMDAR receptors also activate tyrosine phosphatase oxidation (PTP), increasing caspase-3 production as a pro-apoptotic protein leading to cell apoptosis and necrosis. Increased Ca^{2+} due to NMDAR receptor activity can also activate calpains and cathepsins as proteolytic systems in cell death [7]. A decrease in Leydig cell numbers due to brain cell death will affect testosterone production, thus impacting spermatogenesis and the number of spermatogenic cells [24]. The decrease in Leydig cell numbers in the positive control group C (+) leads to narrowing of the interstitial tissue and closer spacing between seminiferous tubules (Figure 1).

One indication of tissue damage due to a decrease in the number of spermatogenic cells can be seen from a decrease in seminiferous tubule diameter [25]. Disrupted spermatogenesis due to reduced testosterone hormone secretion and oxidative stress affects the metabolism of germ cells for proliferation and differentiation [18]. This impacts the testis structure, causing atrophy due to the loss of spermatogenic cells, macroscopic testis damage, and a decrease in seminiferous tubule diameter [26, 27].

The research results show that the positive control group C (+) exposed to 4 mg/gBW/day of MSG showed a significant decrease in seminiferous tubule diameter compared to the negative control group C (-), which only received 0.2 ml/day of CMC Na 0.5%. This is in line with Kalsum et al.'s research, which showed that MSG administration for 30 days in male mice orally could decrease seminiferous tubule diameter and the number of spermatogenic cells [10]. The decrease in seminiferous tubule diameter in the positive control group C (+) results in closer spacing between tubules due to seminiferous tubule degeneration, and spermatogenic cells are arranged in irregular layers (Figure 2). The decrease in seminiferous tubule diameter occurs because MSG exposure can increase the formation of reactive oxygen species (ROS) and suppress testosterone levels during spermatogenesis.

Excessive ROS formation without sufficient endogenous antioxidants leads to oxidative stress in spermatogenic cells. Oxidative stress causes lipid peroxidation in cell membranes and DNA damage [28]. The impact of oxidative stress on the testes leads to spermatogenic cell apoptosis through lipid peroxidation pathways. Apoptosis of spermatogenic cells affects the seminiferous tubule structure and reduces spermatozoa production, ultimately leading to infertility [29]. Damage to these cells reduces the seminiferous tubule diameter, making the lumen diameter appear wider [30].

The treatment groups given *Eleutherine palmifolia* ethanol extract with increasing doses and exposed to 4 mg/gBW/day of MSG showed improvements in Leydig cell numbers and seminiferous tubule diameter compared to the control group (C+). This is consistent with Rohman et al.'s research, which states that the higher the concentration of *Eleutherine palmifolia* extract added, the higher the antioxidant activity it contains [31]. This indicates a protective effect of *Eleutherine palmifolia* ethanol extract in protecting the testes from oxidative stress due to monosodium glutamate exposure.

The T3 treatment group, which was given *Eleutherine palmifolia* ethanol extract at a dose of 120 mg/kgBW/day, showed the best results in maintaining Leydig cell numbers and seminiferous tubule diameter. As seen in Figure 1, the interstitial tissue and spacing between seminiferous tubules widened. Figure 2 also shows seminiferous tubules and spermatogenic cells arranged in an orderly manner. These results are consistent with research showing that *Eleutherine palmifolia* ethanol extract can mitigate free radicals in mice (*Mus muculus*) induced by lead acetate and carbon tetrachloride exposure [15, 32].

Eleutherine palmifolia ethanol extract contains antioxidants such as flavonoids that are highly effective in scavenging free radicals because they contain hydroxyl groups that act as reducers and can donate hydrogen to free radicals [32]. Flavonoids also play a role in suppressing ROS by inhibiting xanthine oxidase and nicotinamide adenine phosphate (NADPH) oxidase activities, as well as binding metals (Fe2+ and Cu2+), preventing chain reactions that can produce free radicals [33]. The mechanism of *Eleutherine palmifolia* ethanol extract administration effectively maintains Leydig cell numbers and seminiferous tubule diameter in mice (*Mus musculus*) exposed to MSG, with the best dose being 120 mg/kgBW/day.

This dose is not yet able to match the number of Leydig cells and seminiferous tubule diameter under normal conditions (C-), so further research is needed on the antioxidant activity of *Eleutherine palmifolia* ethanol extract at different doses in mice (*Mus musculus*) exposed to monosodium glutamate.

5. Conclusion

Based on the research results, the conclusions that can be drawn from this study are as follows: Administration of *Eleutherine palmifolia* ethanol extract can maintain the number of Leydig cells in mice (*Mus musculus*) exposed to MSG, with the best dose being 120 mg/kgBW/day. Administration of *Eleutherine palmifolia* ethanol extract can maintain the seminiferous tubule diameter in mice (*Mus musculus*) exposed to monosodium glutamate, with the best dose being 120 mg/kgBW/day.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

Statement of ethical approval

The study was approved by the Faculty of Veterinary Medicine Animal Ethics Committee of Universitas Airlangga. All variables were considered in accordance with the Ethics Committee related to the animal handling to ensure no discomfort or pain was caused to the animals during sampling(certificate registration number: 1.KEH.081.07.2022.).

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