Influence of *Marsilea crenata* and *Alpinia purpurata* Ethanol Extract on MDA and SOD Testicular Cells of Hyperglycemia Mice

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Abstract

Hyperglycemia can induce testicular damage that leads to male infertility. Herbal plants, such as *Marsilea crenata* and *Alpinia purpurata,* are used for antioxidant defense systems to repair reproductive disorders due to hyperglycemia. This study aims to evaluate the effect of *M. crenata* and *A. purpurata* ethanol extracts on MDA and SOD testes of hyperglycemia mice. This study used a completely randomized design with seven treatment groups (n=4), namely N (control group), H (hyperglycemia mice), Met (hyperglycemia mice given metformin), D1 (0. 09 $mg.g^{-1}$ *M. crenata*), D2 (0.2 $mg.g^{-1}$ *A. purpurata*), D3 (0.09 $mg.g^{-1}$ *M. crenata* + 0.2 $mg.g^{-1}$ *A. purpurata*), and D4 (0.09 $mg.g^{-1}$ *M. crenata* + 0.4 $mg.g^{-1}$ *A. purpurata*). The extracts were administered orally for 17 days. Analysis of testicular MDA and SOD levels was performed by flow cytometry. Data analysis was performed with a one-way ANOVA test and continued with the Tukey test. The results showed that the D4 treatment group, compared to D3, D2, D1, Met, H, and N, showed a better decrease in MDA levels (4.47%) and an increase in SOD levels in the D4 group (4.77%). The research concludes that the combination of 0.09 $mg.g^{-1}$ *M. crenata* and 0.4 $mg.g^{-1}$ *A. purpurata* was an optimal dose to decrease MDA levels and increase SOD levels in the testes of hyperglycemic mice.

Keywords: *Alpinia purpurata*, *Marsilea crenata*, MDA, SOD, testes.

INTRODUCTION

The testes, as the primary male reproductive organ, have the main function of producing sperm and testosterone [1]. This function is supported by a long process of spermatogenesis and steroidogenesis, which underlies the testes as an organ with dense physiological activity [2]. Consequently, it becomes an organ with high mitochondrial oxygen demand, which causes reactive oxygen species (ROS) to accumulate through mitochondrial electron leakage [3]. Although ROS is vital in physiologically tolerable levels for spermatozoa, ROS abundance can initiate oxidative stress (OS), leading to reproductive system disorders [4].

Hyperglycemia is one of the factors that can exacerbate reproductive disorders due to OS. Hyperglycemia is a condition of blood sugar levels exceeding the normal range [5]. Based on reported cases, it was found that hyperglycemia can reduce the birth rate in productive age [6,7]. In this case, 40-50% of them are caused by male infertility [5,8]. In hyperglycemia conditions, ROS can accumulate through several pathways, including activating RAGE by forming advanced glycation end-products (AGEs) [9]. In this condition, antioxidant protection cannot stabilize ROS, which leads to testicular damage [10].

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Testicular cells comprise long-chain ω-6 and ω-3 polyunsaturated fatty acids (PUFAs) [11]. These structures play a role in testes metabolism and may be associated with OS. ROS caused by hyperglycemia can attack PUFAs and induce lipid peroxidation [12,13]. Lipid peroxidation is a chain reaction due to an imbalance of prooxidants and antioxidants [14,15]. Lipid peroxidation can initiate cell, tissue, and organ damage, particularly in the testes. As a result, malondialdehyde (MDA) can be formed as a secondary product of lipid peroxidation. It underlies the evaluation of MDA levels widely used as a biomarker of lipid peroxidation [16].

The human body already has protection to neutralize excess ROS with endogenous antioxidants. Superoxide Dismutase (SOD) is a part of endogenous antioxidants that catalyze superoxide into oxygen and hydrogen peroxide [17]. However, an increase in ROS due to hyperglycemia can initiate a decrease in SOD [18]. It requires preventive approaches by administering exogenous antioxidants. Previous studies have mentioned that administering exogenous antioxidants based on herbs can inhibit spermatozoa and testicular damage due to ROS [19].

Marsilea crenata is a semi-aquatic plant often found in rice fields and considered a weed [20]. It has been reported that *M. crenata* contains phytochemical components such as flavonoids, terpenoids, and saponins [21,22]. These components can potentially be used as a source

of nutrients supporting spermatogenesis [23]. In addition, *Alpinia purpurata*, which is a member of the Zingiberaceae family, has also been reported to contain flavonoids [24]. Both herbal plants are widespread in Indonesia and widely used for food. However, their effect on the antioxidant system in testicular cells with hyperglycemia has not been studied. This study aims to determine the impact of a combination of *M. crenata* and *A. purpurata* ethanol extracts on MDA and SOD testes of hyperglycemia mice.

MATERIAL AND METHOD

Extract Preparation

The dried leaves of *M. crenata* were collected from *Selendang Semanggi*, Surabaya, Indonesia. The sample was converted into powder at UPT Laboratorium Herbal Materia Medica, Batu, Malang, Indonesia (specimen number: 074/ 497/ 102.20-A/2022). *Alpinia purpurata* rhizome powder was purchased from UPT Laboratorium Herbal Materia Medica, Batu, Malang, Indonesia (specimen number: 074/ 496/ 102.20-A/2022).

The powder of each sample was extracted by maceration using a 70% ethanol solvent. Maceration was carried out for 24 hours with a ratio of 1:10 (w:v). The ethanol extracts of *M. Crenata* leaves, and *A. Purpurata* rhizomes were filtered and evaporated using a rotary evaporator [25].

Experimental Design

The protocol of this research was approved by the Animal Care and Use Committee, University of Brawijaya (No. 114-KEP-UB-2022). 28 male BALB/c mice (*Mus musculus*) were obtained from the Faculty of Veterinary Medicine, Airlangga University, Surabaya, East Java, Indonesia. The criteria for experimental animals were 6-7 weeks old with a minimum body weight of 25 g. Mice were kept at room temperature with a 12-hour light-dark cycle and separated from each tail. Mice were fed standard pellets and water *ad libitum*. Acclimatization was carried out seven days before treatment. This study used a completely randomized design with seven treatment groups (n=4) (Table 1). The ethanol extracts of *M. Crenata* and *A. Purpurata* were administered orally for 17 days.

Metformin is a biguanide derivative frequently used in treating hyperglycemia. Metformin can reduce blood sugar levels through the mechanism of insulin sensitivity [26]. In this study, metformin was used as a comparison to represent synthetic drugs commonly used in hyperglycemia conditions.

Table 1. Treatment groups

	Group Description	Extract combination (mg.g^{-1})	
		M. crenata	*A. purpurata*
N	Normal	-	-
H	Hyperglycemia	-	-
Met	Hyperglycemia + Metformin	-	-
D1	Hyperglycemia	0.09	-
D2	Hyperglycemia	-	0.2
D3	Hyperglycemia	0.09	0.2
D4	Hyperglycemia	0.09	0.4

Induction of Hyperglycemia in Experimental Animals

The diabetic agent streptozotocin (STZ) induces hyperglycemia in experimental animals. STZ induction was performed at a dose of 145 mg.kg^{-1} (single high-dose) intraperitoneally after the mice were fasted for 4 hours. STZ was injected in citrate buffer solvent pH 4.5. This solution is recommended for up to 10 minutes to avoid toxicity [25]. Confirmation of blood sugar levels was performed using the glucometer Easy Touch®GCU (Bioptik Technology, Taiwan). Blood sugar ≥ 200 mg.dL-1 is categorized as hyperglycemia mice.

Immunostaining and Flow cytometry

Testicular organs were isolated and homogenized in 3 mL phosphate buffer saline (PBS). Centrifugation was performed at 2500 rpm at 10°C for 5 minutes. The pellet was resuspended and homogenized with 1 mL PBS. Each 100 µL sample was transferred into a microtube and added with 50 µL of CytofixTM to incubate at 4°C for 20 min. The sample was added with 500 µL permeabilization buffer and resuspended. The pellet formed was added with 50 µL FITC anti-MDA and PerCP anti-SOD (BioLegend, USA). Afterward, samples were incubated at 4°C for 20 min. Samples were added 400 µL PBS for running flow cytometry (BD FACSCali-bur, USA) [25].

Statistical Analysis

Statistical analysis was performed using the one-way ANOVA test with a significance value of $p<0.05$ and a confidence level of 95%. If the ANOVA test results show a significant difference, data analysis is continued with the Tukey test. Data analysis was carried out using SPSS 25.0.

RESULT AND DISCUSSION

Confirmation of STZ-induced Hyperglycemia

Mice are confirmed as hyperglycemia if the random plasma glucose ≥200 mg.dL^{-1} [27]. Figure 1 shows the mean blood sugar levels of mice after STZ injection. The N group refers to mice

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not injected by STZ, while the group of H, Met, D1, D2, D3, and D4 refers to mice with induction of STZ. The N group had an average blood sugar level of 108 mg.dL^{-1}. The mean blood sugar levels in H, Met, D1, D2, D3, and D4 were 203 mg.dL^{-1}, 369 mg.dL^{-1}, 225 mg.dL^{-1}, 239 mg.dL^{-1}, 248 mg. dL^{-1}, and 285 mg.dL^{-1}, respectively. The results of this study indicate that treated mice meet the requirements for hyperglycemic experimental animals.

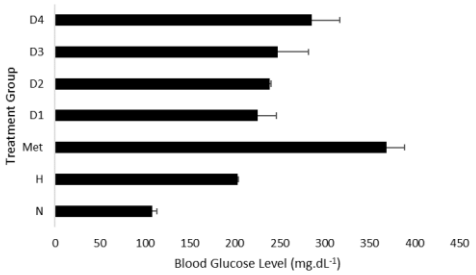

Figure 1. Blood sugar levels post STZ injection. Data are mean ± SD in each group (n=4) with P>0.05.

STZ is a common diabetogenic agent in hyperglycemia model research [28]. STZ induction in experimental animals can selectively damage pancreatic β-cells and increase blood sugar levels [29]. Indeed, STZ can induce Hyperglycemia through processes such as polyol and hexoxamine pathways and glucose autooxidation that causes excess ROS production [30]. Hyperglycemia due to STZ induction can be different in each experimental animal. The use of a single high-dose STZ (145 mg.kg^{-1}) may lead to a rapid increase in blood sugar levels to more than 500 mg.dL^{-1} [29]. It underlies the high blood sugar levels in the Met group (Fig. 1). Hyperglycemia in male primary reproductive organs can initiate ROS accumulation from somatic cells and germ cells. As a result, oxidative stress occurs due to the inability of antioxidant defenses to neutralize ROS, which leads to DNA damage, protein changes, and lipid peroxidation in the testes [31].

The effect of *M. crenata* and *A. purpurata* on MDA Level in Hyperglycemia Testes

MDA is an aldehyde group classified as a secondary product of the lipid peroxidation process [16]. This study showed an increase in the relative number of testicular MDA in mice induced by hyperglycemia characterized by the higher testicular MDA in the H group, with a total of 6.13% compared to the N group, with a total of 5.11%. In sample groups treated with ethanol extract of *M. crenata* and *A. purpurata*, there was a decrease in the relative number of testicular MDA, especially in D2, D3, and D4 when compared to H and Met groups. D2, D3, and D4 groups had testicular MDA levels of 5.65%, 4,62%, and 4.05%. In addition, the amount of testicular MDA in group D4 was found to be significantly different (P<0.05) from that of the H group (Fig. 2).

The increased production of ROS can degrade PUFA and form malondialdehyde (MDA) [32]. ROS represents a reactive free radical that can generate a harmful reduction reaction. ROS is generated in several sources, such as mitochondria, NADPH, cytochrome P450, monocytes, neutrophils, lipoxygenase, and nitric oxide synthase [33]. In the male reproductive system, ROS abundance plays a role in infertility [34]. ROS production in male reproductive organs is classified as internal and external sources.

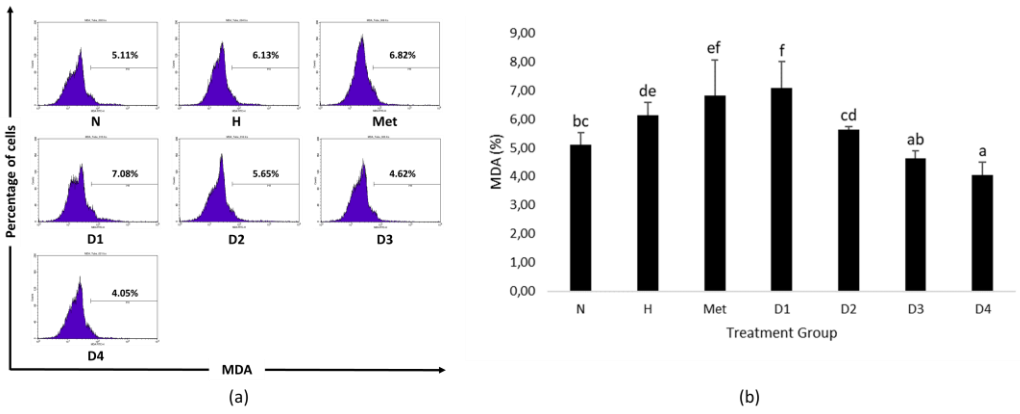

Figure 2. The administration of *M. crenata* and *A. purpurata* extracts reduced testicular MDA levels in hyperglycemia models. (a) Plot of flow cytometry analysis, (b) Histogram of mean statistical results.

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Internal sources of ROS consist of immature spermatozoa, abnormal spermatozoa, neutrophils, and macrophages, which are part of leukocytes. External sources include endocrine-disrupting chemicals (EDCs), smoking, alcohol and drug consumption, and environmental factors such as radiation [35,36]. In addition, degenerative diseases such as diabetes mellitus also contribute to increased ROS in testicular cells [20].

Excessive ROS accumulation is a crucial issue. Testes and their components are considered to be composed of PUFA, which is very susceptible to lipid peroxidation. As a result, impaired testicular cell function, loss of membrane integrity, decreased sperm quality, and induced apoptosis [37]. It is consistent with the increase in testicular MDA levels in hyperglycemia mice as a biomarker of lipid peroxidation. The antioxidant therapy strategy provided in this study was the decrease in MDA levels after treatment with ethanol extracts of *M. crenata* leaves and *A. purpurata* rhizomes. These results align with previous research that showed a decrease in testicular MDA levels in MSG-induced mice after treatment with ethanol extract of *M. crenata* [38]. *M. crenata* is reported to contain genistein, daidzein, and quercetin to reduce MDA levels in the testes [38-40]. In addition, *A. purpurata* is reported to have several active compounds, such as kaempferol, carbohydrates, terpenoids, glycosides, saponins, and flavonoids, that indicate its high antioxidant activity [41].

The Effect of *M. crenata* and *A. purpurata* on Testicular SOD Activity

Testes with PUFA-rich structures have a distinct strategy for protecting themselves from oxidative stress. Under hyperglycemia conditions, moderate autophagy is required to preserve testicular damage by maintaining homeostasis [12,42]. In addition, antioxidant enzymes provide defense for testicular cells in overcoming excess ROS due to hyperglycemia. SOD, as one of the enzymatic antioxidants, plays a role in protecting testicular cells, especially spermatozoa, from oxidative stress and lipid peroxidation [37]. In addition, endogenous antioxidants in spermatozoa are limited compared to other organs [43]. It underlies the importance of providing exogenous sources of antioxidants as a preventive measure to prevent testicular damage due to ROS accumulation [37].

The results (Fig. 3) showed that the administration of the extract improved in relative number of testicular SOD of mice induced by hyperglycemia. The H group had a total SOD of 5.66%, while group N was 3.43%. The increased SOD in the H group compared to the N group occurred as a result of testicular cells' response to maintain their homeostasis. Although it was unable to exceed Met groups, the administration of ethanol extracts of *M. crenata* and *A. purpurata* at all four doses showed an increase in SOD percentage when compared to the normal group.

The results SOD from the four D-groups were 5.46% (D1), 4.70% (D2), 4.70% (D3), and 4.86% (D4), which significantly different from the normal group ($P<0.05$) (Fig. 3b). These results indicate that D4 is the optimal dose for improving SOD activity in hyperglycemia testes. The calculation of the MDA : SOD ratio used to determine the optimal dose of *M. crenata* and *A. purpurata* ethanol extracts on testicular antioxidant molecules in hyperglycemia mice.

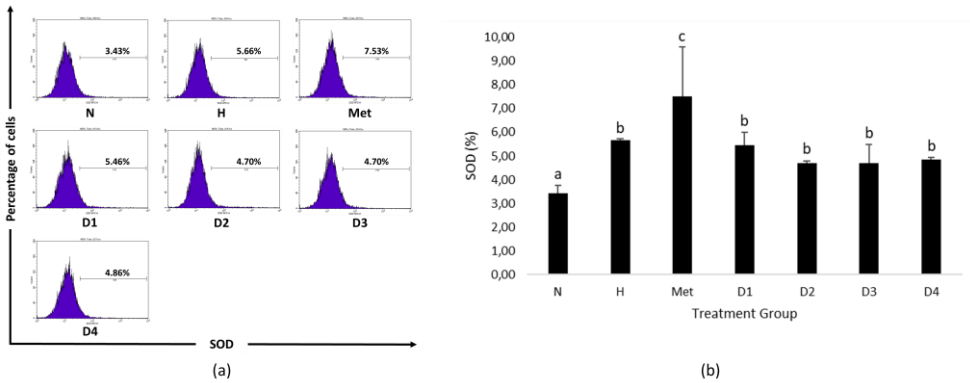

Figure 3. Administration of *M. crenata* and *A. purpurata* extracts increased testicular SOD levels of hyperglycemia models. (a) Plot of flow cytometry analysis, (b) Histogram of mean statistical results.

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The lowest MDA : SOD ratio can indicate the most optimal dose in improving testicular antioxidants in hyperglycemia mice. Through the ratio calculation of the relative number of testicular MDA : SOD, the lowest ratio was obtained in the D4 group.

Marsilea crenata contained phytochemicals in alkaloid, flavonoid, and terpenoid groups. Besides, *A. purpurata* has been reported to contain glycosides, saponins, terpenoids, resins, tannins, and flavonoids. Kaempferol is identified as one of the flavonoids found in *M. Crenata* and *A. purpurata* [41,44,45]. Kaempferol contains anti-inflammatory, anticancer, and antioxidant activities. As an antioxidant agent, kaempferol suppresses the production of superoxide ions and acts as a scavenges in conditions of excessive ROS. Kaempferol is reported to be a therapeutic agent in diabetes mellitus [46,47]. Previous studies showed that kaempferol can increase SOD activity by increasing SOD1 and SOD2 gene expression [48]. Therefore, this study's results align with previous research.

CONCLUSION

This study demonstrated that ethanol extracts of *M. crenata* leaves and *A. purpurata* rhizomes significantly reduced the percentage of testicular MDA in hyperglycemia-induced mice. In addition, the flavonoid content, especially kaempferol, in both herbs enhanced the activity of the SOD antioxidant enzyme. Thus, *M. crenata* and *A. purpurata* are candidates for antioxidant therapy to prevent male infertility in hyperglycemia. Further research related to in silico antioxidant activity mechanisms is needed to strengthen and confirm the results of this study.

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