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The Effect of Areca Nut (*Areca catechu* L.) Ethanol Extract on the Morphology and Histology of Alloxan Induced Rat (*Rattus norvegicus* L.) Testis

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ABSTRACT

Some people traditionally use the areca (*Areca catechu*) nut in Indonesia to cure diabetes; however, information regarding the safety of the areca nut, especially for the reproductive system, still needs to be studied. This study aimed to analyze the effect of administering areca nut ethanol extract on the morphology and histology of testes in alloxan-induced diabetic rats. Twenty-four diabetic rats were divided into six treatments: normal control, alloxan control, 3 treatments of areca nut ethanol extract (100, 200, or 300 mg/kg BW), and metformin 45 mg/kg BW. The treatment was given for 25 days, and on the 26th day, the rats were dissected, and histological preparations were made using the paraffin method and stained with Hematoxylin and Eosin (HE). The results showed that administration of areca nut ethanol extract up to a dose of 300 mg/kg BW was safe for the testis organs and could even increase testis weight, seminiferous tubule diameter, seminiferous tubule germinal epithelial thickness, number of Leydig cells, number of Sertoli cells, number of spermatogonium cells, spermatocytes, spermatids, and spermatogenesis index significantly ($p<0.05$).

Keywords: Alloxan, Areca nut (*Areca catechu* L.), Diebetes mellitus, Testis.

ABSTRAK

Biji pinang (Areca catechu) secara tradisional digunakan oleh sebagian masyarakat di Indonesia untuk mengobati diabetes, namun demikian informasi mengenai keamanan biji pinang khususnya terhadap sistem reproduksi masih perlu diteliti. Tujuan dari penelitian ini adalah untuk menganalisis efek pemberian ekstrak etanol biji pinang terhadap morfologi dan histologi testis tikus yang diinduksi diabetes dengan aloksan. Duapuluh empat tikus percobaan diabetes dibagi ke dalam 6 perlakuan yaitu: kontrol normal, kontrol aloksan, 3 perlakuan ekstrak etanol biji pinang (dosis 100, 200, atau 300 mg/kg BB), dan metformin 45 mg/kg BB. Perlakuan diberikan selama 25 hari dan pada hari ke 26 tikus dibedah dan dibuat sediaan histologi dengan menggunakan metode paraffin dan pewarnaan Hematoxylin dan Eosin (HE). Hasil penelitian menujukkan, pemberian ekstrak etanol biji pinang sampai dosis 300 mg/kg BB aman terhadap organ testis, bahkan dapat meningkatkan berat testis, diameter tubulus seminiferus, tebal epitel germinal tubulus seminiferus, jumlah sel Leydig, jumlah sel Sertoli, jumlah sel spermatogonium, spermatosit, spermatid dan indeks spermatogenesis secara signifikan ($p<0,05$).

Kata kunci: *Aloksan, Biji Pinang (Areca catechu L.), Diabetes melitus, Testis.*

1. Introduction

Untreated diabetes mellitus can cause male infertility due to damage to the structure of the testicular organs [1]. Damage to testicular structures in diabetes occurs due to an increase in ROS. The unbalance of ROS (Reactive Oxygen Species) with the total antioxidant capacity in the body can cause oxidative stress so

that the secretion of LH and FSH will be inhibited [2]. The inhibited secretion of LH and FSH can cause a decrease in the function and number of Leydig and Sertoli cells, resulting in decreased testosterone production. Decreased testosterone production can result in the number of spermatogenic cells being unable to maintain their activity, which can interfere with the process of spermatogenesis [3]. The decrease in the number of spermatogenic cells has a direct impact on the size of the diameter of the seminiferous tubules and the thickness of the germinal epithelium of the seminiferous tubules and can reduce testicular weight [4].

Treatment of diabetes mellitus can be done by taking synthetic antidiabetic drugs [5]. However, synthetic drugs taken over a long period can cause unwanted side effects such as liver problems, nausea, flatulence, and diarrhea [6]. Therefore, individuals with diabetes mellitus (DM) need alternative treatments derived from plants. The use of drugs derived from plants has fewer side effects when compared to synthetic drugs [7]. Areca nuts have been studied to contain secondary metabolite compounds such as alkaloids, tannins, saponins, and flavonoids that can act as antioxidants. Antioxidants in areca nut have activity values above 3.5 µg/ml and are included in the strong antioxidant category [8]. So it can be used as an antidiabetic.

Although ethanol extract of areca nut can be used as a treatment for diabetes, its utilization requires considerations such as dose accuracy, timeliness of use, correctness of ingredients, and accuracy of information, where prolonged use still has undesirable risks to the body [9]. Until now, information regarding the safety of using ethanol extract from areca nuts as an antidiabetic to testicular organs is still very limited. Therefore, it is necessary to conduct empirical research on the effects of the administration of areca nut ethanol extract on the morphology and histology of the testis of diabetes mellitus model rats.

2. Methods

2.1 Materials

The materials used in this research are male Wistar rats aged ± 3 months with a body weight of 150-200 g, feed, wood husk, alloxan, areca nut ethanol extract, metformin 500 mg, aquadest, alcohol, Hematoxylin-Eosin (HE) coloring, dissecting set, gavage, vacuum rotary evaporator, object glass, microtome, tissue processor, and light microscope with camera.

2.2 Making Ethanol Extract of Areca Nut

Make ethanol extract from the areca nut using the maceration method. Areca nuts are split into two parts and dried in the sun without direct sunlight to remove the moisture content. Areca nuts were pulverized with a blender and sieved with a sieve until areca nut symposia were obtained. Areca nut symplisia was weighed at as much as 500 g and then put into a container, and 96% ethanol solvent was added to it, adding as much as 5 liters. Then, closed tightly, stored in a place not exposed to direct sunlight, and left for 3 x 24 hours while stirring occasionally. The extract is filtered using filter paper, and then areca nut filtrate is obtained. Areca nut filtrate was evaporated using a vacuum rotary evaporator at 70ºC and continued thickening with a water bath at 60ºC until a thick extract of areca nut was obtained [10].

2.3 Alloxan Induction in Rats

Alloxan was induced intraperitoneally at a dose of 150 mg/kg BW, as much as 1 ml/rat. On the 3rd day (72 hours) and 7th day (168 hours), the rats' blood glucose levels were measured again to ensure the rats had experienced permanent hyperglycemia. On the 7th day, rats that had blood glucose levels ≥ 200 mg/dl were separated and suitable for use as test animals. Rats with blood glucose levels < 200 mg/dl were re-induced with alloxan. Food and tap water were given ad libitum during induction [11].

2.4 Administration of Areca Nut Ethanol Extract and Metformin

Ethanol extract of areca nut and metformin were given to rats orally using a gavage. Ethanol extract of areca nut was given to rats in treatment groups P1, P2, and P3 at 100, 200, and 300 mg/kg BW doses, while metformin 45 mg/kg BW was given to group P4. Ethanol extract of areca nut and metformin were given daily for 25 days with a 1 ml/individual volume.

2.5 Dissection and Histology Preparation of Testis

In this research, the procedure for making testicular histology preparations consisted of fixation, trimming, dehydration, clearing, infiltration, embedding, cutting, staining, and mounting.

2.6 Data Analysis

The data that has been obtained is analyzed using SPSS 22 software. Normality and homogeneity tests are carried out to assess the normal and homogeneous distribution of data. If the data is normal and

homogeneous (p>0.05), proceed with the One Way Anova test; if p <0.05, then proceed with the post hoc Duncan test.

3. Results and Discussion

3.1 Testicular Morphology

3.1.1 Testicular weight

The results of Post Hoc Duncan analysis showed a significant decrease in testicular weight of diabetic rats (K+) (p<0.05) compared to the normal control group (K-). After being given ethanol extract of areca nut at the highest dose (P3), there was an increase that was not significantly different (p>0.05) from the normal control group (Figure 3.1).

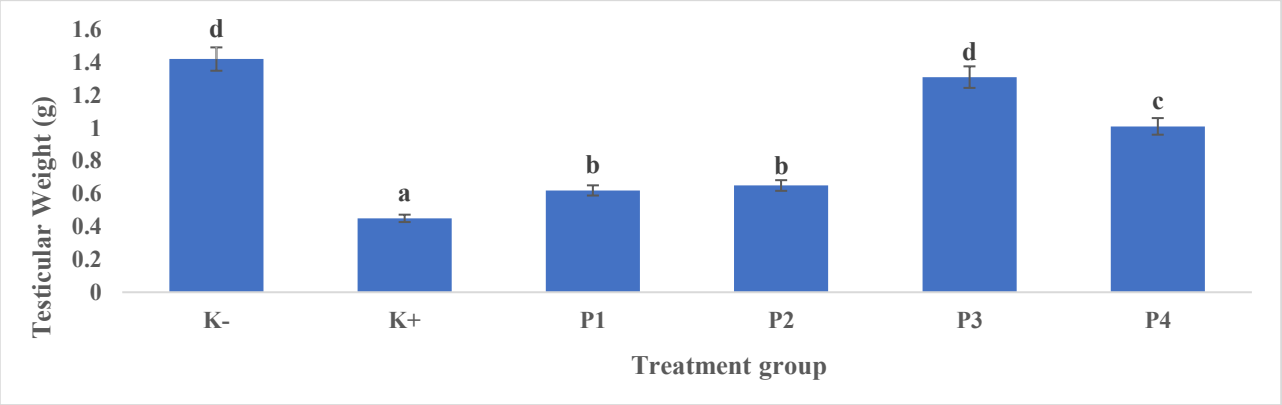


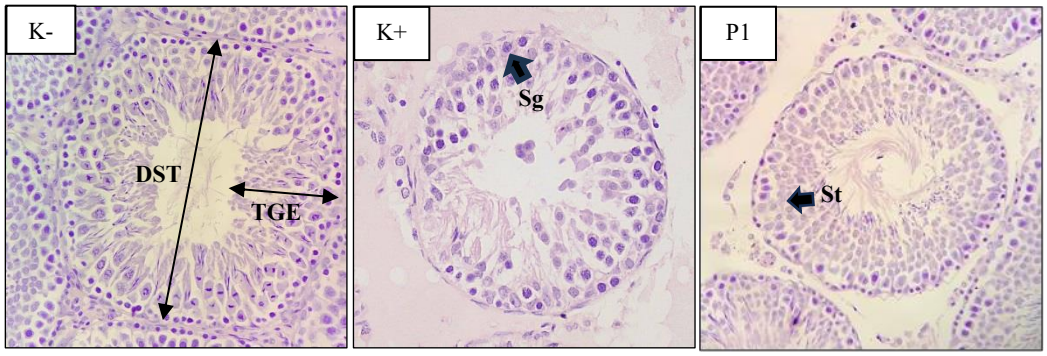
Figure 3.1 Average testicular weight of rats after being treated with various doses of ethanol extract of areca nut. K(-): normal control (without alloxan induction), K(+): positive control (induced alloxan 150 mg/kg BW, without being treated), P1: alloxan 150 mg/kg BW + areca nut extract 100 mg/kg BW, P2: alloxan 150 mg/kg BW + areca nut ethanol extract 200 mg/kg BW, P3: alloxan 150 mg/kg BW + areca nut ethanol extract 300 mg/kg BW, P4: alloxan 150 mg/kg BW + metformin 45 mg/kg BW.

The impact of diabetes mellitus (DM) is an increase in free radicals in the reproductive organs. The high production of free radicals causes a decrease in testosterone levels due to decreased Leydig cell function. If testosterone levels decrease, it will result in the initiation process in Sertoli cells, which play a role in maintaining the existence of germ cells. If this condition continues, it can result in a reduced number of spermatogenic cells so that the diameter of the seminiferous tubules is narrowed, decreasing testicular weight [12].

After being given areca nut ethanol extract, there was an increase in testicular weight in groups P1 to P4. However, the P3 group had testicular means almost close to the normal group (K-). So, it can be seen that the administration of areca nut ethanol extract does not damage testicular morphology but increases testicular weight. Increased testicular weight can occur due to antioxidant content, such as flavonoids in areca nut seeds. Flavonoids can suppress the increase in free radicals that interfere with spermatogenesis. Thus, when the number of spermatogenic cells increases, the size of the seminiferous tubule diameter will increase, and testicular weight will also increase [13]. The increase in seminiferous tubule diameter size directly impacts testicular weight because seminiferous tubules make up 80% of the testicular mass [14].

3.1.2 Testicular Histology

Figure 3.2 shows the results of observations of testicular histology of diabetic rats that have been given an ethanol extract of areca nut seeds at 400x magnification.



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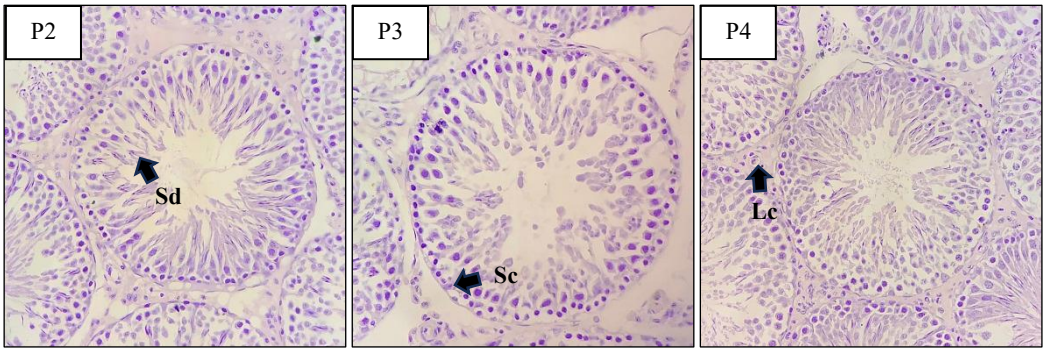

Figure 3.2 Testicular histology of diabetic rats after given ethanol extract of areca nut at 400 x magnification with HE staining. K(-): normal control (without alloxan induction), K(+): positive control (induced alloxan 150 mg/kg BW, without treatment), P1: alloxan 150 mg/kg BW + areca nut extract 100 mg/kg BW, P2: alloxan 150 mg/kg BW + areca nut ethanol extract 200 mg/kg BW, P3: alloxan 150 mg/kg BW + areca nut ethanol extract 300 mg/kg BW, P4: alloxan 150 mg/kg BW + metformin 45 mg/kg BW. Description: DTS: Diameter of the seminiferous tubules, TEG: Thickness of the Germinal Epithelium, Sg: Spermatogonium, St: Spermatocyte, Sd: Spermatid, Sc: Sertoli cells, Lc: Leydig cell.

3.1.2.1 Diameter and Thickness of The Germinal Epithelium of Seminiferous Tubules

From the results of *Post Hoc Duncan* analysis, the diameter of the tubules and the thickness of the germinal epithelium of seminiferous tubules in diabetic rats decreased significantly ($p<0.05$) compared to normal controls. In (P3), there was an increase in the diameter and thickness of the germinal epithelium of seminiferous tubules, which was not significantly different ($p>0.05$) from the normal control after being given ethanol extract of areca nut (Figure 3.3).

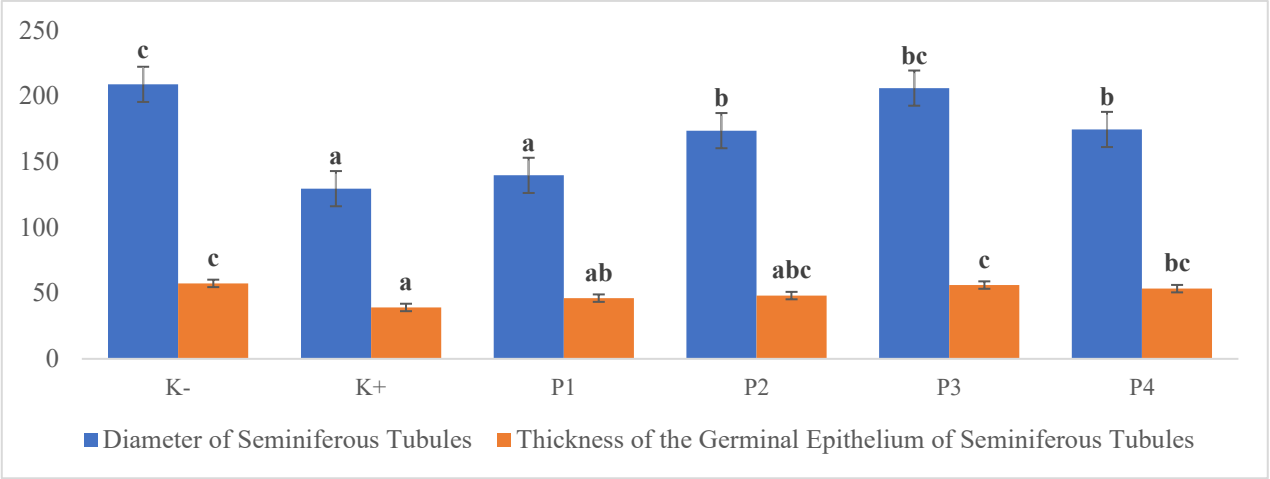

Figure 3.3 Average diameter and thickness of the germinal epithelium of seminiferous tubules of rats after being treated with ethanol extract of areca nut with various doses. K(-): normal control (without alloxan induction), K(+): positive control (induced alloxan 150 mg/kg BW, without being treated), P1: alloxan 150 mg/kg BW + areca nut extract 100 mg/kg BW, P2: alloxan 150 mg/kg BW + areca nut ethanol extract 200 mg/kg BW, P3: alloxan 150 mg/kg BW + areca nut ethanol extract 300 mg/kg BW, P4: alloxan 150 mg/kg BW + metformin 45 mg/kg BW.

In the diabetes mellitus model, rats experience changes in the shape and size of seminiferous tubules, where seminiferous tubules are rarely arranged and shrink. This condition occurs because of the reduced number of Leydig cells and the degeneration of vacuolization, which reduces the production of testosterone hormone, which will directly impact the process of spermatogenesis. If spermatogenesis is disrupted, the number of spermatogenic cells in the seminiferous tubules will decrease, and the diameter of the seminiferous tubules will also decrease [15]. If the size of the diameter of the seminiferous tubules decreases, the thickness of the germinal epithelium of the seminiferous tubules will also decrease [16].

The size of the diameter of the seminiferous tubules and the thickness of the germinal epithelium of the seminiferous tubules at P3 has reached the normal group (K-). Based on the results [17], the ethanol extract of areca nut contains very high secondary metabolite compounds that have an active role as antioxidants. Antioxidant administration can protect the function and structure of testicular cells by protecting the cell plasma membrane from ROS by interrupting the oxidative chain so that the production of ROS is reduced. These antioxidants can change the level of androgens such as testosterone, which is responsible for the process

of spermatogenesis so that if the number of cells that fill the seminiferous tubules increases, it will be able to increase the diameter and thickness of the germinal epithelium of the seminiferous tubules.

3.1.2.2 Number of Testicular Cells

From the results of *Post Hoc Duncan* analysis, the number of testicular cells in diabetic rats decreased significantly ($p<0.05$) compared to normal controls. In (P3), there was an increase in the number of testicular cells that were not significantly different ($p>0.05$) from the normal control after being given ethanol extract of areca nut (Table 3.1).

Table 3.1 Average number of testicular cells of diabetic rats after given ethanol extract of areca nut

Treatment Group	Spermatogenic cell			Leydig cell	Sertoli cell
	Spermatogonium cell	Spermatocyte cell	Spermatid cell		
K-	52.62 ± 2.3^{c}	53.16 ± 2.6^{c}	67.47 ± 7.0^{d}	38.30 ± 4.0^{b}	17.7 ± 17.7^{d}
K+	38.05 ± 6.4^{a}	27.30 ± 4.7^{a}	24.52 ± 7.5^{a}	25.47 ± 6.2^{a}	6.4 ± 6.4^{a}
P1	36.62 ± 2.0^{a}	39.05 ± 5.3^{b}	37.22 ± 5.2^{b}	27.20 ± 5.1^{a}	9.1 ± 9.1^{b}
P2	43.97 ± 2.5^{b}	48.12 ± 2.5^{c}	43.37 ± 1.9^{b}	32.05 ± 1.3^{ab}	12.2 ± 12.2^{c}
P3	50.22 ± 5.0^{c}	53.85 ± 1.3^{c}	67.97 ± 5.1^{d}	37.27 ± 5.2^{b}	17.1 ± 17.1^{d}
P4	49.47 ± 2.2^{bc}	51.05 ± 3.6^{c}	53.45 ± 2.1^{c}	34.15 ± 2.9^{b}	13.0 ± 13.0^{c}

The decrease in the number of spermatogenic cells in diabetic rats (K+) is due to the unavailability of energy sources in the form of glucose for spermatogenic cells to divide due to free radicals resulting from alloxan induction, which results in inhibition of the spermatogenesis process. This free radical might be the cause of abnormalities in the quantity and quality of sperm cells, resulting in infertility [18]. The result of [19], diabetes mellitus can damage sperm through lipid peroxidation and protein oxidation. Oxidative damage initiates sperm plasma membrane destruction, apoptosis, and germ cell death.

Leydig cells have a role in secreting the hormone testosterone, which is needed for germ cells to divide to form spermatozoa [20]. According to [21], the decrease in the number of Leydig cells is due to the entry of free radical exposure so that there is a disturbance in the hypothalamus that secretes GnRH, where there is a decrease in cell division activity, which causes a decrease in the number and function of Leydig cells.

Similarly to spermatogenic cells and Leydig cells, a decrease in the number of Sertoli cells in the testis could indicate the failure of Sertoli cell function to protect germ cells against cell apoptosis. Failure of Sertoli cell function will disrupt spermatogenesis. If Sertoli cell function is impaired, the secretion of ABP (Androgen Binding Protein), nutrient supply, growth factors, lactate, and transferrin will also be impaired, thus inhibiting the process of spermatogenesis [22].

After being given ethanol extract of areca nut for 25 days, it can be seen that there is an increase in the number of spermatogenic cells, Leydig cells, and Sertoli cells. Group P3 is a group that has several testicular cell numbers that are almost close to the normal group (K-). Areca nuts contain secondary metabolites such as flavonoids, alkaloids, saponins, and tannins that can act as antioxidants. According to [23] and [24], the content of secondary metabolites contained in areca nut seeds can protect and maintain the number of Sertoli cells and spermatogenic cells from oxidative stress caused by exposure to free radicals due to diabetes mellitus. In addition, the arecoline content in areca nuts can stimulate the production of the hormone testosterone, which works directly on the Leydig cells of the testis [25]. In this case, the administration of areca nut ethanol extract did not reduce the number of testicular cells but increased the number of diabetic rats' testicular cells.

4. Conclusion

The administration of areca nut ethanol extract at the highest dose (300 mg/BW) had no adverse effect on the testes. This dose increased testicular weight and significantly increased the number of spermatogonia, spermatocytes, spermatid cells, Leydig cells, and Sertoli cells ($p<0.05$).

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