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Effect of Pakkat (Calamus caesius blume) Ethanol Extract on Testis Tissues Histology of Diabetic Rats

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Abstract

BACKGROUND: Diabetes mellitus (DM) is a metabolic disease characterized by elevated blood glucose levels or chronic hyperglycemia. Diabetes has been shown to have an adverse effect on male and female reproductive function and its impact can be seen in the increasing prevalence of infertility. Pakkat is believed to be used as a medicine for DM. The chemical compounds contained in pakkat consist of flavonoids, alkaloids, terpenoids/steroids, saponins, tannins, and glycosides which can be useful for lowering blood glucose levels.

AIM: To investigate the effect of Pakkat extact on Testis Tissues Histology of Male Diabetic Rats.

METHODS: This study used experimental with pretest-post test control group design. This study also used 25 male diabetic rats, that grouped into five groups: Negative control, Positive control, Treatment I (extract dose of 125 mg/ kgBW), Treatment II (extract dose of 250 mg/ kgBW), and Treatment III (extract dose of 500 mg/ kgBW) for two weeks. After that, all rats were sacrificed for resection of testis tissues and these testis tissues were stained hematoxylin and eosin.

RESULTS: Testis histology revealed that all treatment groups had better germinal epithelial than the control negative group. Moreover, the two highest groups (treatment II and III) had better germinal epithelial than the control positive group. A good germinal epithelial is indicated by the presence of many various degrees of developing spermatocytes, supported by some Sertoli cells.

CONCLUSION: Pakkat extract has some phytochemicals, that also has antidiabetic effect and improving the germinal epithelial in Tissue tissue.

Introduction

The incidence of diabetes mellitus (DM) and complications is a major global health problem. The International Diabetes Federation (IDF) estimates that a person in 11 adults aged 20–79 years (415 million adults) had DM globally in 2015 [1]. The World Health Organization (WHO) predicts that the rate of diabetic patients in Indonesia will increase from 8.4 million in 2000 to 21.3 in 2030. Like WHO, the IDF also estimates that the number of people with DM will increase from 7 million in 2009 to 12 million in 2030. These report still show an increase in the number of people with DM 2–3 times in 2030 [2].

The three main classifications of diabetes are type 1 diabetes, type 2 DM, and gestational DM. Since 2000, the IDF has reported on the national, regional, and global incidence of diabetes. In 2009, an estimated 285 million people had diabetes (type 1 and 2 diabetes combined, increasing to 366 million in 2011, 382 million in 2013, 415 million in 2015, and 425 million in 2017) [3]. DM is a metabolic disorder characterized by increased blood glucose levels due to abnormalities in insulin secretion, insulin action, or both [4]. Some risk factors for diabetes were age (45–69 years), marital status, hypertension, obesity, and family history [5]. Other factors that may increase diabetic prevalence include lifestyle, diet, work environment, exercise, and stress [6]. Some symptoms in diabetic patients are polyuria and polydipsia, weight gain, or loss. Long-term complications that diabetics can cause include kidney failure, heart disease, macrovascular stroke, microvascular retinopathy, and neuropathy [7].

Pakkat is young rattan, a typical food for the Mandailing community in South Tapanuli, North Sumatra. Pakkat is believed to be used as a medicine for DM. Thus, it is very popular in the public setting. A previous study reported the presence of some phytochemicals, including flavonoids, alkaloids, terpenoids/steroids, saponins, and tannins [8].

Methods

This experimental study used a pretest-posttest randomized control group design. This study

was performed in March 2021 at the Pharmacology Laboratory of Universitas Prima Indonesia. All protocol study has been approved by Health Research Ethics Committee from Universitas Prima Indonesia.

This study used male Wistar rats aged 7 weeks and weighed 175–200 g because the Wistar rats are Mammalia. Therefore, the impact on treatment may not be much different from other mammals. In addition, this study used male wistar rats due to economic considerations, the rat's ability to live up to 2–3 years, and the ease of handling the rats [9], [10]. The following formula determined the number of male Wistar rats:

$$(n-1)\ (t-1) \geq 15$$
$$(n-1)\ (5-1) \geq 15$$
$$4\ n-4 \geq 15$$
$$4\ n \geq 19$$
$$n \geq 4{,}75 = 5$$

t: Number of treatments

n: Number of repetitions

Based on the formula above, it can be seen that the number of male Wistar rats was 5 male Wistar rats per group. Thus, the total number of male Wistar rats used in this study was 20 male Wistar rats [11].

The extract of Pakkat (*Calamus caesius* Blume) was first powdered and macerated with 96% ethanol in a ratio of 1:7 for 3 days. After that, it was filtrated. The filtrate was collected for evaporation. Meanwhile, the residue was re-macerated in the same way. Furthermore, all filtrate is concentrated with a rotary evaporator and a water bath to obtain a concentrated Pakkat ethanol extract [12].

The concentrated Pakkat ethanol extract was undergone a phytochemical screening for flavonoids (NaOH), phenols (FeCl3), alkaloids (Mayer, Dragendroff), saponins (Foam), tannins (FeCl3), and steroids/terpenoids (Libermann Burchard's, Salkowski's) [13].

1. Flavonoid Test

Take 2 mL of the extract, add five drops of NaOH, then add a few drops (five drops) of dilute HCl. Formed in a yellow solution with NaOH and turned yellow to colorless with dilute HCl, indicating the presence of flavonoids [14].

2. Alkaloid Test

Take 1 mL of extract, put it in a test tube, add a small quantity of 2N HCl solution, heat it, and add Mayer's solution until a white or yellow lump precipitate forms; Dragendrof has a brown to black precipitate [15].

3. Saponin Test

Take 0.5 g of extract and mix it with 10 mL of hot water. Once cool, stir vigorously for 10 s to produce foam. Then add 1 drop of 2N HCl to observe the strength of the foam. The presence of foam indicates the presence of saponin [16].

4. Tannin Test

Take 10 mg of extract dissolved in 45% ethanol. Then boil the test tubes for 5 min and add 1 mL of $FeCl_3$ solution to each test tube. A green-to-black color change indicates the presence of tannins in the extract [17].

5. Steroid/Terpenoid Test

Take 0.5 mL of extract, add 2 mL of acetic anhydride, and then 2 mL of sulfuric acid. In Liebermann–Burchard's, the formation of a brown ring color in the top solution indicates the presence of steroids and terpenoids [18]. At Salkowski's, take 3 mL of extract, add 1 mL of chloroform, and a few drops of concentrated sulfuric acid. If the formation of a reddish-brown precipitate indicates the presence of terpenoids [19].

GC-MS analyzed the obtained Sunkist ethanol extract based on the procedure of the NRE Laboratory manual. This analysis was performed by GC-MS spectroscopic detection using an electron ionization system with an ionization energy of 70 eV. Pure helium gas was used as carrier gas at a constant flow rate of around 1 mL/min. The initial oven temperature was 50°C and was increased gradually with a rate of 3°C/min until 150°C; then, it was held in isothermal condition for 10 min and raised to 300°C at 10°C/min. Around a microliter of concentrated extract that had been diluted by ethanol with a ratio of 1:100 was injected in the split mode with a split ratio of 120: 1. After that, the relative percentage of chemical constituents in Sunkist ethanol extract was expressed as a percentage by peak area normalization [20].

All male Wistar rats were adapted to a laboratory setting for a week before the study and then given food and access to drinking with the aim that the experimental animals did not experience stress and were in good condition [21]. Rats fasted for 12–18 h, and then, alloxan was induced at a dose of 150 mg/kg BW. Alloxan was dissolved in water and injected intraperitoneally, and then blood glucose levels were monitored for 14 days. If the blood glucose level is >200 mg/dL, it is considered diabetes and can be used in research [22].

The 1% NaCMC Solution was prepared by mixing 1 g of NaCMC with 100 mL of aquadest to make a 1% NaCMC solution. Sprinkle evenly on the aquadest, wait about 15 min, then stir until completely dissolved. Using a probe, it is given to animals as much as 1 mL/150 g of body weight [23].

Diabetic rats were treated for 14 days (2 weeks). Rats were randomly grouped into five groups, including:

1. Negative Control: given NaCMC orally
2. Positive Control: given metformin at a dose of 45 mg/kgBW
3. Treatment I: Given extract dose of 125 mg/kgBW
4. Treatment II: Given extract dose of 250 mg/kgBW

5. Treatment III: Given extract at a dose of 500 mg/kgBW.

Each group received treatment orally once a day for 2 weeks [24]. Blood was taken by taking tail vein blood, about 1 mL of blood volume. Blood Glucose levels were checked by rubbing the rat's tail with 70% alcohol, then cutting the tail's tip to form a small wound, and then dropping the blood on the glucometer strip [25].

After 14 days, all rats were sacrificed by chloroform inhalation in a closed room. After that, all rats were fixed into paraffin blocks, and the abdomen wall was incised vertically to expose the organ in the abdomen cavity. Then, the testis was dissected and washed into normal saline. Moreover, the washed testis organ was kept in 10% buffer formalin solution until it was processed to stain [26], [27].

Results

This study used concentrated Pakkat ethanol extract, which was extracted by maceration method using 96% ethanol extract as the solvent. This extract then underwent a phytochemicals screening and the result is described in Table 1 [12], [28], [29].

Table 1: Phytochemical screening of pakkat ethanol extract

Phytochemicals	Reagent	Result
Trepenoid/steroid	Libermann	+
	Salkowski	+
Saponin	Aquadest	+
Flavonoid	Shinoda Test (Mg+HCl)	+
	Pb (CH3COO) 2 1–5%	+
	NaOH	+
Tannin	FeCl3	+
Alkaloid	Mayer	-
	Dragendroff	-

Table 1 shows that the Pakkat ethanol extract contained some phytochemicals compounds, including flavonoids, saponins, tannins, and steroids/terpenoids. After that, the concentrated Pakkat ethanol extract underwent GC-MS analysis to identify the various content in the Pakkat ethanol extract and the eesult of GC-MS analysis result is described in Table 2.

Table 2: GC-MS analysis of pakkat ethanol extract

RT	Quality	Compounds	Level (%)
4.449	38	3-trans-Methoxy-2-cis- Methyl-1R-Cyclohexanol	12.87
27.534	35	Butanoic Acid, Silver (1+) Salt	5.87
28.162	72	Hexadecanoid Acid, Methyl Ester	3.79
29.348	95	7,10-Octadecadienoic Acid, Methyl Ester	7.28
30.651	62	Trans-13-Octadecenoic Acid	5.19
30.872	92	(9E,12E)- 9,12-Octadecadienoyl - Chlodride	6.67
31.148	94	2-Aminoethanethiol Hydrogen Sulfate (Ester)	8.15
31.568	83	1,2-Benzenedicarboxylic Acid	20.47
32.444	91	Oleic Acid, Propyl Ester	20.57
38.539	98	Diosgenin	27.10
38.891	97	.gamma. – Sitosterol	8.34

Table 2 shows that Pakkat ethanol extract had some phytochemical contents; the highest compound level was diosgenin (27.10%) and the lowest was hexadecanoid acid, methyl ester (3.79%).

Blood glucose level

Ethanol extract is the most widely used preparation in the initial study of plant pharmacology. Because early research like this does not yet know, in which compounds are responsible for the pharmacological effects of plants, so it is necessary to use general solvents that can dissolve secondary metabolites of different polarities [30]. This study was divided into five treatment groups which were tested on the blood glucose levels of male Wistar rats before alloxan induction, after alloxan induction, 7 days after treatment and 14 days after treatment which were divided into Control (-), Control (+) groups with metformin administration, Treatment I with extract dose of 125 mg/kgBW, Treatment II extract dose 250 mg/kgBW, and Treatment III extract dose 500 mg/kgBW. Comparison of These Blood Glucose Level were described in Table 3.

Data are displayed as Mean ± SD; p-values were obtained from one-way ANOVA; Superscript. Description: Control (-) (NaCMC), Control (+) (Metformin), Treatment I (Extract dose 125 mg/kgBW), Treatment II (Extract dose 250 mg/kgBW), and Treatment III (Extract dose 500 mg/kg BW).

From the data in the table above, it can be seen that the blood glucose levels of rats after 14 days of treatment, blood glucose levels will decrease significantly, which can be seen from $p < 0.05$. The negative control group had the highest blood glucose level after 14 days of treatment, namely, 556.00 ± 46.15 mg/dL and the lowest was the extract 250 mg/kgBW, which was 160.20 ± 72.75 mg/dL. Based on research, it is said that flavonoids can reduce blood glucose levels, can reduce blood glucose levels through two mechanisms, namely, intra- and extrapancreatic and play an important role as antioxidants and antidiabetic drugs by inhibiting free radicals [31]. This study also evaluates the histology of testes tissue in all groups and the histology of testes tissues which are described in Figure 1.

Based on Figure 1, it can be seen that the all-treatment groups had better germinal epithelial than the control negative group. Moreover, the two highest groups (Treatments II and III) had better germinal epithelial than the control positive group. A good germinal epithelial is indicated by the presence of

Table 3: Comparison of treatment groups on rat blood glucose levels

Group	Control (-)	Control (+)	Treatment I	Treatment II	Treatment III	p-value
Before induction	106.40 ± 14.64	108.60 ± 22.77	100.00 ± 17.34	109.20 ± 6.73	104.00 ± 17.59	0.910
After induction	420.80 ± 173.51	417.20 ± 139.07	472.60 ± 80.20	525.40 ± 80.20	483.52 ± 135.85	0.664
7th days after induction	452.80 ± 57.77	270.60 ± 147.79	401.20 ± 217.29	334.20 ± 156.10	286.60 ± 10.94	0.353
7th days after induction	556.00 ± 46.15	162.80 ± 42.79	216.60 ± 128.74	160.20 ± 72.75	514.60 ± 42.10	0.000

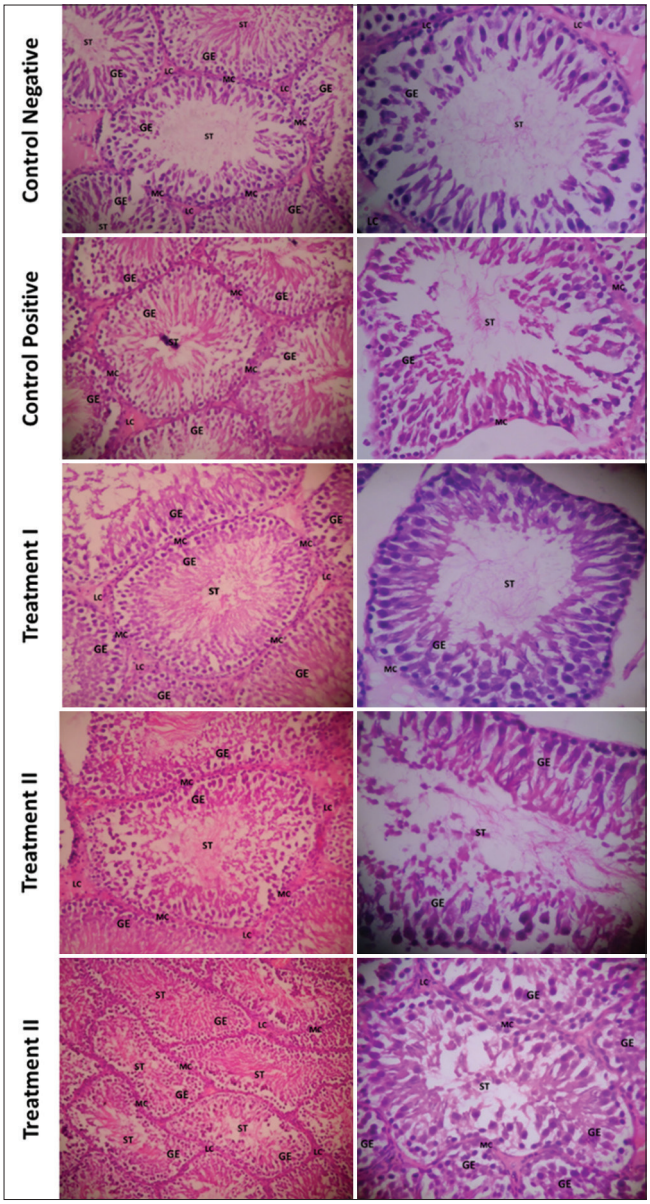

Figure 1: Histology of Testes Tissue in All Groups. Magnification: 100× (Left) and 400× (Right). Stain: Hematoxylin and Eosin. GE: Germinal epithelial; SE: Seminiferous tubule (ST); LC: Leydig cells in interstitial stroma; MC: Myeloid cells in lamina propria

many various degrees of developing spermatocytes, supported by some Sertoli cells.

Discussion

Pakkat or young rattan is believed to have efficacy in the treatment of diabetes [32], because it obtained secondary metabolites in the form of flavonoids, alkaloids, saponins, tannins, and steroids/terpenoids in its content [8].

In positive controls, metformin was given because it is a biguanide anti-diabetic drug that is widely used to treat non-insulin-dependent diabetes [33]. The mechanism of action of metformin is to lower blood glucose levels by increasing glucose transport to muscle cells [34].

Pakkat has various phytochemical content that is responsible for many pharmacological effects. Flavonoids prevent damage to the spermatozoa membrane so that the process of spermatogenesis is not disturbed and rescues the germinal epithelial in the testis tissue [31]. Flavonoids can also bind to receptors estrogen alpha in the testes and epididymis so that it has an estrogenic function and can work in conjunction with testosterone to aid the maturation of spermatozoa in germinal epithelial in testis tissue [31].

Flavonoid antioxidants can prevent oxidative stress due to high reactive oxygen species (ROS). This antioxidant is also a preventive defense that cuts the reaction ROS with free radicals, which can improve the motility of spermatozoa on DM [31]. To neutralize free radicals, antioxidants are needed that can bind directly to free radicals, prevent ROS formation, convert ROS into less toxic, and repair damaged cells and tissues [31].

Conclusion

Overall, it can be concluded that Pakkat (*C. caesius* Blume) has some phytochemicals, including flavonoids, terpenoids/steroids, saponins, and tannins. It has an antidiabetic effect by reducing blood glucose levels and improving the germinal epithelial in testis tissue.

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