Effect of sikkam (Bischofia javanica Blume) ethanolic extract on the quality and quantity of hyperglycemic rat sperm

[Efecto del extracto etánolico de sikkam (Bischofia javanica Blume) sobre la calidad y cantidad de esperma de rata hiperglúcémico]

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Abstract

Context: Hyperglycemia causes diabetes mellitus (DM), abnormal metabolism, oxidative stress, and chronic complications such as impotence. Hyperglycemia causes testicular atrophy and stromal cell, seminiferous tubular damage, and spermatogenic cells. Bischofia javanica Blume is a plant that is used for the treatment of various chronic conditions and has traditionally been the plant of Indonesia as a diabetes medicine.

Aims: To determine the effect of B. javanica extract on the increase in the quality and quantity of sperm of hyperglycemic rats.

Methods: The treatment groups consisted of; G0: negative control (-), G1: positive control (DM: alloxan induction + standard feed), G2-G4: DM + 300, 600 and 900 mg/kg BW of B. javanica leaves ethanol extract, respectively, and G5: DM + glibenclamide 0.5 mg/kg BW. Rats were dissected, and then the testes were taken to analyze sperm quantity and quality and immunohistochemistry.

Results: There was a significant difference (p<0.05) in testes volume, sperm concentration and sperm motility in hyperglycemic rats. The decrease in caspase 3 expression and apoptosis was accompanied by an increase in the dose of the highest B. javanica ethanol extract, and it was seen that testicular histology in groups G4 (900 mg/kg) and G5 (glibenclamide) could improve testicular histology like in the control group (G0).

Conclusions: B. javanica can improve the quality and quantity of hyperglycemic rats’ sperm and also reduce apoptosis via caspase 3 in the histology of testis.

Keywords: apoptosis; caspase 3; hyperglycemia; immunohistochemistry; plant extracts; sperm.

Resumen

Contexto: La hiperglycemia causa diabetes mellitus (DM), metabolismo anormal, estrés oxidativo y complicaciones crónicas como impotencia. La hiperglycemia causa atrofia testicular y células estromales, daño tubular seminífero y células espermato génicas. Bischofia javanica Blume es una planta que se utiliza para el tratamiento de diversas afecciones crónicas y tradicionalmente la gente de Indonesia la ha utilizado como medicamento para la diabetes.

Objetivos: Determinar el efecto del extracto de B. javanica sobre el aumento de la calidad y cantidad de espermatozoïdes de ratas hiperglúcémicas.

Métodos: Los grupos de tratamiento consistieron en; G0: control negativo (-), G1: control positivo (DM: inducción de aloxano + alimentación estándar), G2-G4: DM + 300, 600 y 900 mg/kg de peso corporal de extracto etánolico de hojas de B. javanica, respectivamente, y G5: DM + glibenclamida 0,5 mg/kg de peso corporal. Se disecaron las ratas y luego se tomaron los testículos para analizar la cantidad y calidad de los espermatozoïdes y la inmunohistoquímica.

Resultados: Hubo una diferencia significativa (p<0,05) en el volumen de los testículos, la concentración de espermatozoïdes y la motilidad de los espermatozoïdes en ratas hiperglúcémicas. La disminución de la expresión de caspasa 3 y apoptosis fue acompañada por un aumento en la mayor dosis del extracto, y se observó mediante histología una mejoría testicular en los grupos G4 (900 mg/kg) y G5 (glibenclamida) similar al grupo control (G0).

Conclusions: B. javanica puede mejorar la calidad y cantidad de esperma de ratas hiperglúcémicas y también reducir la apoptosis vía caspasa 3 en la histología de testículos.

Palabras Clave: apoptosis; caspasa 3; esperma; extractos de plantas; hiperglucemia; inmunohistoquímica.
INTRODUCTION

Metabolic disorders in diabetes mellitus can cause dysfunction of the male reproductive system (Zhao et al., 2020). Diabetes mellitus (DM) is a metabolic disorder that increases high blood glucose levels (hyperglycemia), which can cause cardiovascular disease, neuropathy, nephropathy, retinopathy, and male impotence (Barkabi-Zanjani et al., 2020). Hyperglycemia in DM patients causes disturbances in carbohydrate, fat, and protein metabolism and causes chronic complications such as kidney failure, nerves, impotence, increased uric acid cholesterol levels and aortic disorders (Landon et al., 2020; Ilyas et al., 2021). Hyperglycemia also causes diabetic vascular neuropathy, abnormal metabolism, oxidative stress, and insulin resistance syndrome. It reduces the secretion of sex hormones, including gonadotropins, luteinizing hormone, testosterone, and follicle-stimulating hormone (Shi et al., 2017). Hyperglycemia causes testicular atrophy and stromal cell, seminiferous tubal damage, and spermatogenic cells (Imani et al., 2021).

In the male reproductive system, it is known that male reproductive dysfunction has occurred with changes in testicular morphology. DM causes a decrease in mass in the head, body, and caudal. In addition, the number of spermatids is reduced in the tubules, germ cell population and stereocilia (Sahin et al., 2019). DM also causes inflammation and clumping of epithelial cells and lipid vacuolization (Barkabi-Zanjani et al., 2020). Reactive oxygen species (ROS) are the main cause of cell damage in sperm cells due to microcirculation disorders that can cause insulin resistance (IR). Long-term stimulation of blood sugar leads to an upregulation of the number of mitochondria in tissue cells, and lipid peroxidation causes damage to the antioxidant defense system (Sahin et al., 2019). DM has the potential to cause male infertility because DM can affect the expression of genes involved in sperm DNA repair, resulting in high levels of nuclear DNA fragmentation, deletion of mitochondrial DNA with changes in the mitochondrial respiratory chain, decreased sperm motility, and affects caspase expression and apoptosis (Condorelli et al., 2018).

The main cause of DNA damage is ROS-mediated apoptosis and is most common in infertile male spermatozoa (Bisht et al., 2017). Apoptosis is characterized by the disintegration of chromatin leading to DNA fragmentation, which is a biological process essential for proper germ cell formation and maintenance of the germ cell-Sertoli ratio in the testes (Asadi et al., 2021). Direct or indirect ROS-mediated damage can lead to single or double-stranded fragments as well as abnormal caspase activity and apoptosis (Asadi et al., 2021).

Each plant consists of several main phytochemical compounds that are useful for intrinsic defense and several direct or indirect therapeutic potentials, which serve to synthesize and develop modern drugs. *Bischofia javanica* Blume (family Phyllanthaceae) is a plant used to treat various chronic conditions such as inflammation, tuberculosis, ulcers, fractures, and dislocations. This plant is also used to treat diabetes, sore throat, and nervous disorders (Hutahaean et al., 2021). *B. javanica* leaves ethanol extract also contains quercetin and gallic acid as antidiabetic (Kituyi et al., 2018). This plant has traditionally been used by the people of Indonesia (especially the Pakpak tribe in North Sumatra province) as a diabetes medicine (Silalahi et al., 2018). That’s because the leaves on plants produce ten main phytochemicals viz. beta-amyrine, ursolic acid, betulinic acid, chrysoberyl, quercetin, friedelan-3-one, beta-sitosterol, fisetin, cynaroside and triacontane (Chowdhury et al., 2020).

This study aims to determine whether the extract of *B. javanica* extract can increase the quality and quantity of sperm of hyperglycemic rats (*Rattus norvegicus*) and to analyze testicular histology through protein caspase and apoptosis after administration of this plant. This research is expected to be developed into a potential drug to prevent diabetic men’s impotence in the future.

MATERIAL AND METHODS

Preparation of *B. javanica* leaves ethanol extract

*B. javanica* leaves were collected from Simalungun Regency, Indonesia (2°59’17.5″ N, 98°52’29.3″ E). The plant was diagnosed and authenticated by means of the Head of Botany in the Universitas Sumatera Utara and deposited into the Medanean Botanical Herbarium (Registration range 5395/MEDAN/2020).

Extraction was conducted using the maceration method with 96% ethanol as the solvent. A total of 1 kg of fresh *B. javanica* leaves were dried and then were obtained 270 g of ethanol extract of *B. javanica* leaves. The drying process was based on the methods used for drying *Rhodomyrtus tomentosa* and *Zanthoxylum acaanthopodium* as described previously (Situmorang et al., 2019a; 2019b; 2021a; 2021b; 2021c), so that the phytochemical content in this herb was not lost due to heat.

*B. javanica* leaves were soaked in a large glass jar, and 1000 mL of 96% ethanol was added. The glass jar was closed tightly, and the pinnacle of the jar was

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protected with aluminum foil and incubated for three
days, including daylight, and the soaked leaves were
stirred each day. After three days, the marinade was
filtered and stored in a distinctive bottle. Then, the
filtering consequences were evaporated using an elec-
tric-powered heater. Once thick, its miles were posi-
tioned right into a steam cup wrapped in aluminum
foil overnight, and then its miles were allowed to
evaporate once more until the extract was thick. B. javanica leaves were then moved into the pattern bot-
tles for extraction. Furthermore, the simplified waste
was macerated once more using 96% ethanol. This
was performed for up to three macerations, and as a
result, we received 100 g of B. javanica ethanol extract.

Animal handling

Thirty-six male Wistar rats were acclimatized for
14 days. The treatment of male rats was adapted to
the laboratory environment for two weeks and placed
in a cage with a constant room temperature (25.0 ±
3.0°C), a humidity of 35-60%, and light-dark cycles for
12 hours. Male rats were fed maize and pellets on a
dietary basis ad libitum and given free water access.
The rats were placed in a plastic case measuring 40
cm × 30 cm. Alloxan induction was performed with
the aid of intraperitoneal injection. Before activation,
alloxan was dissolved first using 0.9% NaCl. Alloxan
injection was administered at a dose of 160 mg/kg
BW. Rats that have been triggered with DM were
used for treatments. The experiments were approved
by the Health Research Ethics Committee of USU Medan (Number: 0256/KEPH-FMIPA/2021).

Study design

This experimental study was performed using a
non-factorial, completely randomized design (CRD).
The experiments were performed in six groups of
remedies within this study. The remedy groups con-
sisted of G0: negative control (-), G1: positive control
(DM: alloxan induction + standard feed), G2: DM +
300 mg/kg BW of B. javanica leaves ethanol extract,
G3: DM + 600 mg/kg BW of B. javanica leaves ethanol
extract, G4: DM + 900 mg/kg BW of B. javanica leaves
ethanol extract; and G5: DM + 0.5 mg/kg BW
glibenclamide orally. After the rats experienced hy-
perglycemia due to alloxan induction, rats were ad-
ministered an ethanol extract of B. javanica leaves for
14 days orally. Rats were dissected through the dislo-
cation method (ketamine injection before), and then
the testes were removed for analyzing the sperm
quantity and quality and immunohistochemistry.

Collection and analysis of quality and quantity of
rats sperm

The spermatozoa were collected by massaging the
cut vas deferens using a watch glass containing 0.9%
NaCl solution, and the motility test was performed to
assess the percentage of motile spermatozoa. After
obtaining the number of motile (moving) spermato-
zoa to decipher the total number above, they were
divided by the number of dead spermatozoa obtained
by applying Goerge's solution. Sperm viability was
calculated through making smear preparations from
the stock solution, stained with Giemsa, and then
visualized under a microscope. Data on sperm count,
abnormality, and viability were analyzed using one-
way ANOVA followed by the BNT test (Samour,
2004).

Immunohistochemistry of caspase 3

The testes were fixed in neutral buffered formalin
(10%), processed into paraffin blocks, and then sec-
tioned to a thickness of 5 µm and stained with hema-
toxylin and eosin (H&E), and immunohistochemical
staining with anti-caspase 3 antibody. For immuno-
histochemical staining for caspase 3, the avidin-
biotinperoxidase method was used to detect the activated
caspase 3 as a marker of apoptosis (Resendes et al.,
2004). Brown staining was considered a superb re-
response in the cytoplasm and nucleus. Then, depara-
finization and rehydration were performed. Antigen
retrieval was performed through boiling in citrate
buffer inside the microwave. Endogenous peroxidase
blocked the use of H2O2, and non-specific serum Tris
buffer (10%) was added for 20 min at room tempera-
ture and then incubated with rabbit anti-caspase 3
 polyclonal antibody (catalog no RP096, Diagnostic
Biosystems; USA) diluted 1/100 at room temperature
for 120 min. The slides were then incubated with a
biotinylated polyclonal secondary antibody and incu-
bated with avidin-biotin-peroxidase complex solution
(LSAB 2 Kit; Dako). The response was visualized us-
ing 3,3'-diaminobenzidine tetrachloride to the testicu-
lar tissue. The samples were covered with a pitcher
pawl and observed under a microscope.

Tunel assay

Paraffin-embedded testes tissue was further sec-
tioned to reduce the size using a microtome with a
thickness of 4 μm. TUNEL assay was performed using
detection kit (Promega, Cat # G7130; USA). The
testes tissue inside the slide was immersed in clean
xylene for 5 min. The slides were rehydrated with
multilevel ethanol and washed with NaCl 0.85% and
PBS for 5 min. After rehydration, the cells were incu-
bated for 15 min at room temperature with proteinase
K (20 μg/mL). The last labeling response was
changed by assessing the response upon adding rTdT to the slides in a damp room (37°C for 1 h). The response of the rTdT enzyme was assessed by immersing the slide in a buffer at room temperature. The slides were washed for 5 min with PBS. Endogenous peroxidase was blocked using 0.3% hydrogen peroxide in PBS. A streptavidin-horseradish peroxidase solution was added to the tissue and incubated at room temperature. The chromogenic substrate DAB was then added to the slide. All slides were dehydrated with graded ethanol and washed with 100% xylene for 5 min thrice (Dutta et al., 2012).

Statistical analysis

Statistical analysis of the data was performed using the Statistical Package for Social Science 22.0 (SPSS 22). Comparison between groups (sperm quantity and quality) was conducted using ANOVA, while the immunohistochemical analysis and TUNEL assay were analyzed using the Kruskal Wallis test. Data were presented as the mean ± standard deviation. Statistical significance was set at p<0.05.

RESULTS

Volume of testis after given B. javanica leaves

There was a significant difference (p<0.05) in the analysis of testicular volume analyzed using the ANOVA one-way test. The control group (G0) exhibited the highest average testicular volume. The lowest testicular volume was observed in the DM group, which was on the standard feed (G1). However, the difference was not significant between groups G2–G5. Fig. 1 indicates that diabetes without treatment can lead to a decrease in testicular volume.

Sperm concentration of diabetic rats

A significant difference (p<0.05) in the sperm concentration value was analyzed using the one-way ANOVA test. The control group (G0) exhibited the highest average testicular volume (Fig. 2). The lowest testicular volume was observed in the DM group, which was on the standard feed (G1). However, the difference was not significant between groups G2 and G3 compared to the G1 group. The highest sperm concentration was observed in the control group because this group was without diabetes, followed by the G5 group, who were administered with glibenclamide at a concentration of 0.5 mg/kg BW. Since the drug could control high blood sugar levels, there was no significant decrease in sperm volume.

Sperm motility of diabetic rats

A significant difference (p<0.05) in sperm motility was analyzed using the one-way ANOVA test. The control group (G0) exhibited the highest mean sperm motility, followed by G2–G5 groups. The lowest testicular volume was observed in the DM group, which was on the standard feed (G1). Motility is the ability of sperm cells in semen to move and swim to reach the egg. Healthy sperm are usually assessed based on their ability to move 25 μm per second. Fig. 3 indicates that diabetes affects sperm motility in males.

Sperm viability of diabetic rats

No significant difference (p>0.05) in the sperm viability value was analyzed using the one-way ANOVA test. Viability indicates the survival of a sperm that is weak and prone to death. Although semen contains a lot of sperm, not all sperm are viable. Fig. 4 indicates that diabetes does not affect sperm viability.
A significant difference (p<0.05) in caspase 3 expression was analyzed using the Kruskal-Wallis test. The G1 group exhibited the highest mean caspase 3 expression as this DM group was only on the standard feed (G1). The lowest caspase 3 expression was observed in the control group (G0), followed by groups G4 (DM + B. javanica ethanol extract 900 mg/kg BW) to G5 (DM + glibenclamide 0.5 mg/kg BW). Hematoxylin and eosin staining and caspase-3 staining (Fig. 5) in the control group (G0) confirmed regular histological capabilities, and in the G1 group, disrupted interstitial tissue was confirmed. A basement membrane was visible surrounding the tubules that enclosed the myoid cells. The tubules were found to be covered with seminiferous epithelium-shaped Sertoli cells and a layer of spermatogenic cells consisting of spermatogonia, spermatocytes, spermatids, and spermatozoa (Fig. 5). Spermatogonia with dark nuclei were visible, resting at the basement membrane. Primary spermatocytes were the most important cells within the spermatogenic collection and displayed a nucleus with broken chromatin. Near the lumen were spermatids, the first (round) and last (long). Spermatozoa were found to be present in the lumen of the tubules, and Leydig cells were found in the interstitial tissue between the tubules. The decrease in caspase 3 expression was accompanied by an increase in the dose of B. javanica ethanol extract, and it was observed that the testicular tissue in groups G4 and G5 (Figs. 5 and 6) was almost normal, as in the control group (G0).

### DISCUSSION

Fig. 1 indicates that diabetes without treatment can cause a decrease in testicular volume. Oxidative stress caused by diabetes mellitus can damage the testes as an organ that plays a role in spermatogenesis, thereby reducing the production of spermatozoa, which in turn leads to impotence. Gallic acid and quercetin in B. javanica leaves are antidiabetic agents that suppress hyperglycemia by inhibiting active glucose transport, such that the group administered these herbs did not exhibit a significant decrease in testicular volume (Chowdhury et al., 2020; Hutahaean et al., 2021).
Figure 5. Caspase-3 expression of spermatogenic cells in the testis tissue of diabetic rats treated with saline solution, B. javanica extracts, and glibenclamide.

G0: Control, G1: DM + standard feed, G2: DM + 300 mg/kg BW of B. javanica ethanol extract, G3: DM + 600 mg/kg BW of B. javanica ethanol extract, G4: DM + B. javanica ethanol extract 900 mg/kg BW and G5: DM + glibenclamide 0.5 mg/kg BW. Red arrow; caspase 3 positive. Magnification: 400x.

Figure 6. Caspase-3 expression of germ cells of diabetic rats treated with saline solution, B. javanica extracts, and glibenclamide.

Data are represented as mean ± standard deviation (n = 6). Different letters indicate statistically significant differences between bars (p<0.05) using the one-way ANOVA test. G0: Control, G1: DM + standard feed, G2: DM + 300 mg/kg BW of B. javanica ethanol extract, G3: DM + 600 mg/kg BW of B. javanica ethanol extract, G4: DM + B. javanica ethanol extract 900 mg/kg BW and G5: DM + glibenclamide 0.5 mg/kg BW.

Figure 7. Caspase-3 expression of germ cells of diabetic rat testis treated with saline solution, B. javanica extracts, and glibenclamide.

Data are represented as mean ± standard deviation (n = 6). Different letters indicate statistically significant differences between different groups (p<0.05) using the one-way ANOVA test. G0: Control, G1: DM + standard feed, G2: DM + 300 mg/kg BW of B. javanica ethanol extract, G3: DM + 600 mg/kg BW of B. javanica ethanol extract, G4: DM + B. javanica ethanol extract 900 mg/kg BW and G5: DM + glibenclamide 0.5 mg/kg BW.
The concentration of spermatozoa (Fig. 2) relies on spermatogenesis within the seminiferous tubules. If spermatogenesis usually occurs, a regular variety of spermatozoa may be produced. Conversely, during spermatogenesis, if there is a disturbance, the improvement of spermatogonia cells is impaired and could affect the variety of spermatozoa produced. The decrease in the number of spermatozoa after alloxan treatment is thought to be due to the effects of stress caused by high insulin levels, which activated the endocrine system on the hypothalamic-pituitary-adrenal (HHA) axis, thereby resulting in the disruption of the release of the neurohormonal CRH and a decrease in the number of spermatogonia cells. This occurs due to decreased secretion of LH, FSH, and testosterone. Lack of LH, FSH, and testosterone directly inhibits spermatogonia proliferation and eventually interferes with the process of spermatogenesis. Gallic acid, which acts as an antidiabetic, is closely related to its antioxidant properties (Brohi and Huo, 2017). Antioxidant compounds in B. javanica leaves (groups G2 to G5) neutralize cells experiencing oxidative stress by donating hydrogen atoms (Hutahaean et al., 2021).

Spermatozoa movement is influenced by flagella, which move longitudinally in a rhythmic manner between the posterior and anterior tubules. Spermatozoa comprise the axoneme (Brohi and Huo, 2017). Normal spermatozoa in humans move in a straight line and are affected by the temperature and pH. High temperatures increase sperm motility. Improved plasma membrane integrity and increased intracellular energy levels enhance spermatozoa movement. Many factors affect sperm motility (Fig. 3) and viability (Fig. 4), including the duration in epididymis, morphology, physiology, spermatozoa biochemistry, flagella, agglutination, antibodies, viscosity, pH, temperature, fluid, immunology, and oxidative stress. Hyperglycemia reduces the secretion of sex hormones, including gonadotropins, luteinizing hormone, testosterone, and follicle-stimulating hormone (Shi et al., 2017). Hyperglycemia causes testicular atrophy and damages stromal cells, seminiferous tubules, and spermatogenic cells (Imani et al., 2021).

Apoptosis is a physiological process that regulates the number of cells in the testicular tissue and eliminates germ cells damaged during spermatogenesis. However, excessive apoptosis leads to impaired reproductive function in males. In Figs. 7-8, it is shown that diabetes can lead to an increase in sperm cell apoptosis in the testicular tissue. The strong expression of caspase-3 in the cytoplasm, nucleus of tubular cells, and Leydig cells indicated alloxan-induced apoptosis in mouse testes. Caspase-3 is a key mediator of apoptosis (Sarhan, 2018). Two apoptotic pathways, including extrinsic (receptor pathway) and

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intrinsich (mitochondrial pathway), lead to the activation of the effector caspase-3, which cleaves the intracellular substrates, thereby resulting in drastic morphological and biochemical changes during apoptosis (Sarhan, 2018). The expression of caspase-3 in the testicular cell nuclei and dark-stained irregularly shrunken nuclei was observed in group G1 (Figs. 6-7).

ROS and the resulting oxidative stress can cause cell death by triggering the two apoptotic pathways. A decrease in caspase 3 expression and apoptosis in DM rats treated with alloxan could be because B. javanica exhibits antioxidant activity by inhibiting alpha-amylase, which is an important digestive enzyme that hydrolyzes starch into maltose and then breaks it down into glucose. Therefore, the glucose levels in the case of diabetes can also be reduced using alloxan. The apoptotic process in germ cells can also be triggered by exposure to specific plant secondary metabolites of certain cells (Situmorang and Ilyas, 2018). Therefore, in this study, the increased expression of caspase-3 in group G1 was mainly due to alloxan-induced oxidative stress.

CONCLUSION

This study demonstrated that the ethanol extract of Bischofia javanica can improve the quality and quantity of hyperglycemic rat sperm and reduce apoptosis via regulating caspase 3 in the tissues of the testis. The ethanol extract of Bischofia javanica could be analyzed to the development as a potential drug to prevent impotence in diabetic men in the future.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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