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Effect of *Ruellia tuberosa* L. leaf extract on germinal epithelium thickness and seminiferous tubule diameter in the testes of alloxan-induced white rats

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ABSTRACT

Diabetes Mellitus (DM) is a pathological condition that leads to metabolic disturbances, affecting the health of both human and animals. *R. tuberosa* leaf extract, which contains antioxidants such as saponins, carotenoids, flavonoids, and phenols, has the potential to neutralize free radicals and reduce oxidative stress, thereby mitigating testicular tissue damage. This study aimed to evaluate the effect of *R. tuberosa* leaf extract on germinal epithelium thickness and seminiferous tubule diameter in the testes of alloxan-induced white rats. This experimental study used a Completely Randomized Design (CRD) with white rats as the animal models. The treatment groups included P0 (normal control), K- (diabetic control), K+ (50 mg/kg BW of metformin), P1 (200 mg/kg BW of *R. tuberosa* leaf extract), P2 (400 mg/kg BW of *R. tuberosa* leaf extract). Testicular organ samples were collected on day 14. Histopathological samples were stained with Hematoxylin-Eosin and examined under a trinocular microscope at 100X magnification. Data on germinal epithelium thickness and seminiferous tubule diameter were analyzed using ANOVA, followed by Duncan's test. The group receiving *R. tuberosa* leaf extract (200 mg/kg BW) exhibited the highest mean germinal epithelium thickness, while the diabetic control group showed the lowest value. Similarly, the group treated with *R. tuberosa* leaf extract (400 mg/kg BW) showed the highest seminiferous tubule diameter, and the lowest was the diabetic control group. The study indicated that the administration of *R. tuberosa* leaf extract at doses of 200 mg/kg BW and 400 mg/kg BW significantly improved germinal epithelium thickness and seminiferous tubule diameter compared to the 50 mg/kg BW dose of metformin treatment.

Introduction

Diabetes mellitus (DM) is a pathological condition that leads to metabolic disturbances, affecting the health of both human and animals (Solikhah *et al*., 2024). According to the International Diabetes Federation (IDF), there are approximately 537 million cases of diabetes mellitus (DM) worldwide among individuals aged 20-79 years (Sun *et al*., 2022). DM cases are also commonly observed in animals from the Canidae family, with the data from Banfield Pet Hospital's State of Pet Health report showed an 80% increase in canine diabetes cases in the United States between 2006 and 2016. DM is characterized by hyperglycemia, a persistent increase in blood glucose level caused by insulin secretion disorders, which can disrupt the metabolism of proteins, carbohydrates, and fats (Banday *et al*., 2020).

Hyperglycemia and metabolic dysfunction negatively impact various biochemical processes in the body, one of which is the increased production of intracellular Reactive Oxygen Species (ROS). The imbalance in ROS production can occur under diabetes condition which can significantly contributes to cellular damage. The increased level of ROS can lead to oxidative stress in vital cellular components, including proteins, lipids, and nucleic acids, ultimately resulting in cell death in various organs (Solikhah and Solikhah, 2021).

The imbalance of ROS can affect organs within the reproductive system, one of which is the testes. This condition can lead to functional damage in the testes, including a reduction in the secretion of reproductive hormones such as testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH). It can also cause structural damage to the testicular organ, such as injury to the germ cells in the seminiferous tubules, fibrosis, vascular damage, and morphological changes in the testes (Darbandi *et al*., 2018).

The seminiferous tubules are the primary morphological structures found in the testes and are crucial in spermatozoa production. This organ consists of two main types of cells, which are germ cells and Sertoli cells. Germ cells play a role in the formation of spermatozoa, starting from spermatogonia and maturing into fully developed spermatozoa. Sertoli cells have a crucial role in supporting and facilitating spermatogenesis by providing nutrition, protection, and creating an optimal environment for germ cells. Sertoli cells also have another function which is to regulate the production of reproductive hormones (Esener *et al*., 2017). Animals experiencing ROS imbalance due to diabetes can lead to structural damages and alterations in the components of the seminiferous tubules. The imbalance of ROS level can also result in DNA mutations and cell membrane damage, which affects the normal morphology of the seminiferous tubules (Darbandi *et al*., 2018). Chronic inflammation and oxidative stress caused by diabetes can trigger lipid peroxidation in the blood vessels of the testes, characterized by an increase in Malondialdehyde (MDA) level and the number of free radicals. This condition can lead to a decrease in germ cell production and cell death, resulting in a reduction in the thickness of the germinal epithelium and can directly affect the decrease in the diameter of the seminiferous tubules (Altoé *et al*., 2014).

Diabetic patients are advised to undergo pharmacological therapy by taking medications to control blood sugar level, one of which is metformin (Flory and Lipska, 2019). This medication is a biguanide derivate that is widely used and beneficial in managing complication of diabetes and glucose metabolism. Metformin is taken orally and works by inhibiting the reduction of insulin production and enhancing insulin sensitivity (Khairullah *et al*., 2021). Metformin has side effects including nausea, vomiting, diarrhea, gastrointestinal intolerance, and lactic acidosis (Flory and Lipska, 2019). In addition, in some cases, the combination of metformin with other antidiabetic medications such as glibenclamide and pioglitazone can lead to hypoglycemia (Solikhah *et al*., 2022). Long-term administration of commercial diabetes medications carries risks such as

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acute kidney failure and an increased risk of myocardial infarction. Many developing countries use complementary and alternative treatments due to the high cost of commercial medications (Khairullah *et al*., 2021). The discovery of medicinal plants for diabetes treatment has proven to be beneficial, easily accessible, and has fewer side effects compared to commercial medications (Solikhah *et al*., 2020).

Ruellia tuberosa L. is a plant from the Acanthaceae family which distributed across several countries in Asia, including Indonesia. Phytochemical studies have shown that the leaves of this plant contain antioxidants, such as saponins, carotenoids, flavonoids, and phenols, which can neutralize free radicals by reducing oxidative stress level that is a major cause of testicular tissue damage (Safitri *et al*., 2021). According to research conducted by Safitri *et al*. (2021), the aqueous extraction of *R. tuberosa* root at doses of 200 and 500 mg demonstrated hypoglycemic activity with a 25.35% reduction in blood glucose level in white rats. Histopathological examination of the pancreas, liver, and kidneys showed preventive effects against pancreatic beta cell damage, liver complications, and kidney fibrosis.

Based on the research by Utami *et al*. (2020), the plant *Cinnamomum burmannii* which contains free radical neutralizer such as flavonoids and tannins, reduces the side effects of diabetes mellitus (DM) on testicular histopathological damage, including the diameter of seminiferous tubules and the number of Leydig cells. Previous studies that led to this research highlight the potential of *R. tuberosa* plant in lowering blood glucose level and providing preventive effects against cellular damages in the male reproductive system. This study focuses on observing the germinal epithelium thickness and seminiferous tubules diameter induced by alloxan as the diabetes inducer, allowing for a deeper investigation into testicular histomorphometry.

This study is expected to provide a comprehensive understanding of the potential of *R. tuberosa* leaves in the treatment of diabetes mellitus (DM) and its effects on the reproductive organs. It aimed to offer a scientific basis for the development of alternative diabetes therapies and their impact on reproductive health.

Materials and methods

Ethical approval

This study was conducted under ethical approval number 2.KEH.098.06.2023 obtained from Faculty of Veterinary Medicine, Universitas Airlangga Campus C, Surabaya.

Tools and Materials

The materials used in this study include the leaves of *R. tuberosa* which were collected from Banyuwangi District, Banyuwangi Regency, East Java Province. *R. tuberosa* leaves were purposively selected without compatison to plants from other regions. The research materials include male white rats (*Rattus novergicus*), *R. tuberosa* leaves, CP593 feed, baby corn, wood dust, alloxan, metformin, formalin 10%, ethanol 96%, absolute alcohol at concentrations of 80%, 90%, and 95%, xylol, liquid paraffin, acetate buffer solution, NaCl 0.9%, and Carboxymethyl Cellulose (CMC).

The tools used in this study include glucometer (EasyTouch® GCU), EasyTouch® blood glucose test strips, yellow top vacutainers, oven, stainer, beaker glass, aluminium foil, OneMed® syringes, feeding tube, gloves, wooden cages and wire mesh, surgical instruments, pins, needles, trinocular microscope (Nikon Eclipse 200), surgical board, and feeding and drinking containers.

Preparation of Ruellia tuberosa L. leaf extract

The preparation of *R. tuberosa* leaf extract began with selecting fresh leaves, which were washed under running water. The leaves were cut into 4 cm pieces, then oven-dried at 40ºC for 72 hours before being ground into simplicia using an electric grinder. For extraction, the maceration method was done over 5 consecutive days using a 1:10 ratio of simplicia to solvent, 50 g to 500 mL of 96% ethanol. During the maceration process, the extract was stored in a dark, airtight container away from direct sunlight, and stirred every 6 hours. Afterward, the filtrate was filtered using filter paper and concentrated using a rotary evaporator at 50ºC and 120 rpm. The thick extract was stored in a Beaker glass covered with aluminium foil in a refridgerator at 4ºC to prevent degradation (Solikhah *et al*., 2020). A 0.5% CMC solution was used as the solvent to dissolve the extract to the appropriate dose (Luo *et al*., 2021).

Experimental animals

The white rats were adapted for 7 days. During this period, the white rats were given 20 grams of CP593 feed and 20 grams of baby corn, along with *ad libitum* access to drinking water.

Research design

This study is an experimental research of a Completely Randomized Design (CRD) using white rats as test subjects. After a 7-day adaptation period, type 1 diabetes mellitus was induced by a single intraperitoneal injection of alloxan at a dose of 150 mg/kg BW, dissolved in 0.9% NaCl (Solikhah *et al*., 2022). For 3 consecutive days following the injection, the white rats were provided standard feed and 10% sucrose solution *ad libitum* to prevent hypoglycemic conditions. Blood glucose levels were measured three days post-injection by collectiong blood samples from the tail vein after the white rats were fasted for 12 hours. White rats with fasting blood glucose levels ≥ 200 mg/dL were considered as diabetic and received the extract and metformin therapy for 14 days. The testicular organs of the white rats were collected for analysis on day 14. This study used a total of 25 male white rats, which were randomly divided into five treatment groups, with five rats per group, as follows:

Group 1: Consists of 5 white rats without diabetes induction, medication, or *R. tuberosa* leaf extract.

Group 2: Consists of 5 white rats induced with diabetes using a single dose of alloxan.

Group 3: Consists of 5 white rats induced with diabetes using alloxan, followed by therapy using metformin at a dose of 50 mg/kg BW for 14 days.

Group 4: Consists of 5 white rats induced with diabetes using alloxan, followed by therapy using *R. tuberosa* leaf extract at a dose of 200 mg/kg BW for 14 days.

Group 5: Consists of 5 white rats induced with diabetes using alloxan, followed by therapy using *R. tuberosa* leaf extract at a dose of 400 mg/kg BW for 14 days.

Histopathological examination of the testes

The testicular organs of the white rats were collected on day 14 after treatment administration. Euthanasia was performed through intraperitoneal injection of xylazine at 10 mg/kg BW and ketamine at 100 mg/kg BW. Testicular organs collection was performed using a scalpel blade. Surgery was conducted by making an incision in the lower abdomen until the testicular organs were visible, then the testes were removed by cutting the spermatic cord. The organs were then lifted, cleaned from the connective tissue and fat, and stored in sample containers containing formalin 10% solution to ensure proper fixation of the tissue.

The tissue preparation was carried out using the paraffin method on the left testis (testis sinister). The testicular organ was fixed in formalin 10% solution, followed by trimming with a microtome to obtain tissue sections approximately 4 mm in thickness. The tissue was then dehydrated through graded alcohol concentrations starting from 70%, 80%, 95%, and 96%. After dehydration, the tissue underwent a clearing process us-

ing xylol. The clearing process aims to make the tissue transparent. Following the clearing process, the embedding process was performed by immersing the tissue in liquid paraffin using a paraffin bath. The tissue block was then sectioned with a microtome to a thickness of 4 µm, and the tissue slides were floated on a water bath before being transferred onto the object glass. The tissue was stained using Hematoxylin-Eosin (Gamde *et al*., 2023).

Histopathological morphometric measurement

The microscopic appearance of the testes was observed and measured using Nikon Eclipse E200 trinocular microscope with NIS-Elements software at 100X magnification. The morphometric measurement of the seminiferous tubule diameter was performed by drawing the longest distance connecting the outermost edge of the basal lamina of the seminiferous tubule, passing through the central point. Measurements were taken at two perpendicular lines, with the first diameter measurement being perpendicular to the second diameter measurement. The results were calculated to determine the average (Moffit *et al*., 2007). The morphometric measurement of the germinal epithelium thickness was taken from the spermatogonium layer to the spermatid layer, measured across five fields of view from five seminiferous tubules. The results were calculated to determine the average (Kumar and Nagar, 2014).

Data analysis

The analysis of the seminiferous tubule diameter and germinal epithelium thickness data was performed using SPSS 23. The data were first tested for normality using the Shapiro-Wilk test, and if ($p>0.05$), the data were considered to be normally distributed. Subsequently, a one-way analysis of variance (One-Way ANOVA) was conducted to assess significant differences among the treatment groups, followed by Duncan's multiple range test to compare the mean values between the treatment groups (Ukwubile *et al*., 2023).

Results

The testes observed in this study were limited to the left (sinister) testes only to reduce treatment variability. The adaptation period for the rats was 7 days, followed by administration of alloxan for 1 day and a 3-day waiting period post-injection. The treatment with *R. tuberosa* extracts and drug was carried out for 14 days. The measurements of the seminiferous tubule diameter and the germinal epithelium thickness were performed using a trinocular microscope equipped with NIS-Elements software by Nikon at 100X magnification, to obtain the overall morphology of the seminiferous tubules stained with Hematoxylin-Eosin. Measurements were taken from 5 fields of view, with one seminiferous tubule observed per field. Normality testing was performed using the Shapiro-Wilk test to assess data distribution. The results of the normality test indicated that the seminiferous tubule diameter data followed a normal distribution ($p>0.05$). Homogeneity testing showed that the data for the seminiferous tubule diameter (0.506) and the germinal epithelium thickness (0.096) had a p-value > 0.05, indicating homogeneity. The seminiferous tubule diameter and germinal epithelium thickness data were then subjected to ANOVA, followed by Duncan's test, as significant differences were found in both the germinal epithelium thickness and germinal tubule diameter.

The results of the ANOVA and Duncan's test for the variables of seminiferous tubule diameter and germinal epithelium thickness, as presented in Appendix 12, showed an ANOVA p-value of 0.000 which indicates $p<0.05$, meaning there are significant differences among the groups, supporting the acceptance of H1 and the rejection of H0. Therefore, it can be concluded that all variables exhibit significant differences. The analysis was further conducted using Duncan's test, in which based on the data of seminiferous tubule diameter, showed that group P1 is not significantly different from group P2, but is significantly different from groups P0, K-, and K+. The order of mean seminiferous tubule diameter from lowest to highest is as follows: diabetes control, normal control, metformin 50 mg/kg BW, *R. tuberosa* leaf extract 200 mg/kg BW, and *R. tuberosa* leaf extract 400 mg/kg BW. Based on the Duncan's test for germinal epithelium thickness, the data indicated that group P2 is not significantly different from group P1, but is significantly different from groups P0, K-, and K+. The highest mean of germinal epithelium thickness was observed in the group receiving *R. tuberosa* leaf extract with a dose of 200 mg/kg BW, while the lowest was in the diabetes control group.

Microscopic observations of the seminiferous tubules in the testes analyzed using a trinocular microscope, revealed differences among the treatment groups. The P0 group (normal control) after 14 days of treatment showed a relatively high mean seminiferous tubule diameter (244.19±23.23) and germinal epithelium thickness (68.72±6.11). In this group, the spermatogenic cells were organized, starting from the basal lamina, with a clearly defined basal lamina boundary and numerous spermatozoa were observed within the lumen of the seminiferous tubules. In contrast, the K- group (diabetic control) exhibited a lower mean seminiferous tubule diameter (221.18±22.81) and germinal epithelium thickness (62.54±6.84). In this group, the spermatogenic cells forming the epithelium of the seminiferous tubules appeared sparse, and the germ cell cycle was not clearly visible due to structural changes in the spermatogenic cells. Additionally, empty spaces were observed in the lumen of the seminiferous tubules.

Discussion

Alloxan can increase the production of ROS which can have both direct and indirect effects on germ cells. The induction of diabetes using alloxan has shown negative effects on the histopathological structure of the testes, including a reduction in the germ cell population, the formation of lipid vacuolization, inflammatory infiltration, and morphological alterations of germ cells (Solikhah *et al*., 2023).

The study results showed that the K- group (diabetic control without treatment) experienced a significant reduction in the mean of seminiferous tubule diameter and the mean of germinal epithelium thickness compared to the P0 group (normal control without extract or drug treatment). Oxidative stress can disrupt germ cells, Leydig cells, and Sertoli cells (Köprülü *et al*., 2022). The death of Sertoli cells which have function as

Table 1. Average Diameter and Germinal Epithelium Thickness of Seminiferous Tubules in The Testes of White Rats (Rattus norvegicus) Across Different Treatment Groups. Mean values followed by different letters (a, b, c) indicate significant differences.

	Seminiferous Tubule Diameter (µm)	Germinal Epithelium Thickness (µm)
Normal control (P0)	244.19±23.23^{b}	68.72±6.11^{b}
Diabetic control (K-)	221.18±22.81^{a}	62.54±6.84^{a}
Metformin (K+)	258.58±19.67^{c}	76.32±9.07^{c}
R. tuberosa Leaf Extract 200mg/kg (P1)	283.44±21.93^{d}	88.50±7.97^{d}
R. tuberosa Leaf Extract 400mg/kg (P2)	284.77±20.85^{d}	86.54±7.47^{d}

Data are expressed as Mean±SD. Mean values followed by different letters (a, b, c) indicate significant differences.

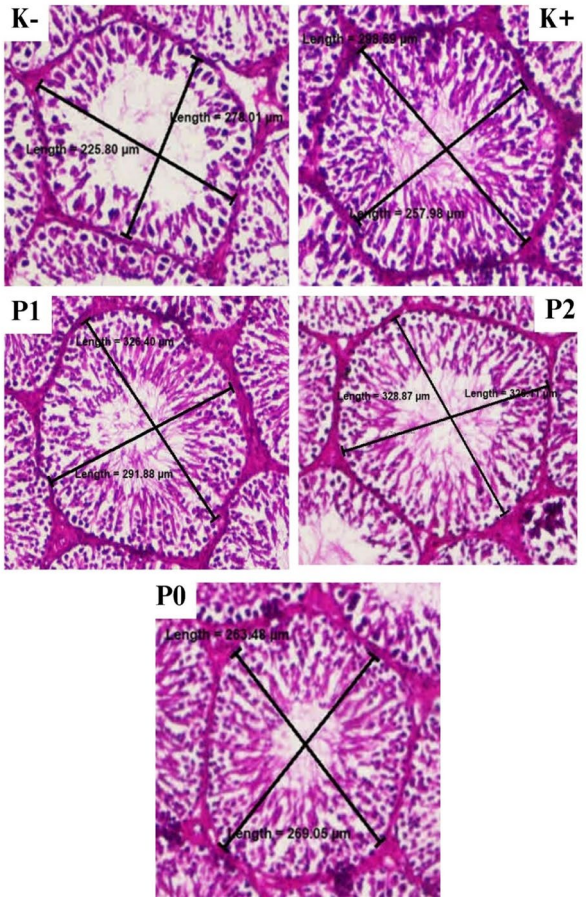

Figure 1. Measurement of Seminiferous Tubule Diameter with HE Staining at 100X Magnification Using a Trinocular Microscope.

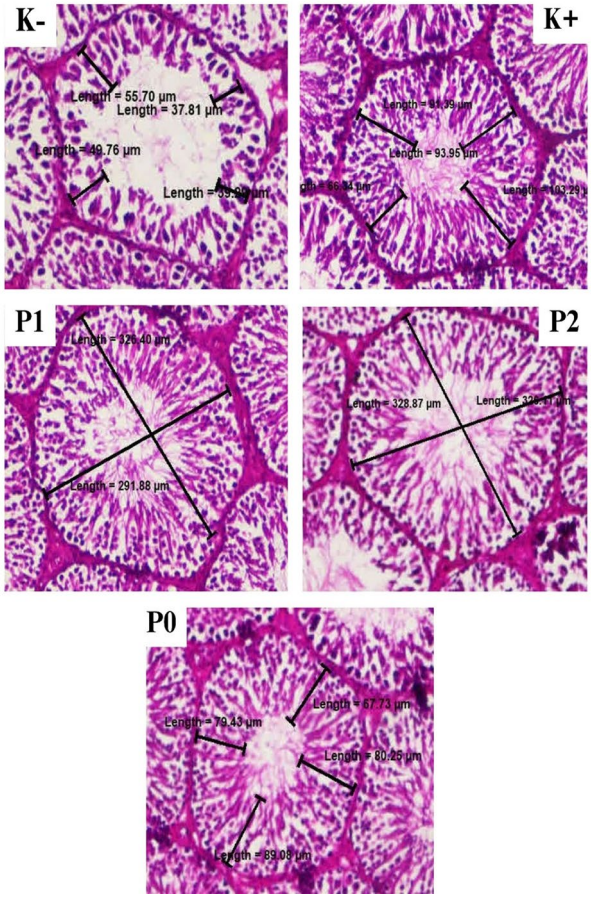

Figure 2. Measurement of Germinal Epithelium Thickness in Seminiferous Tubules of White Rats with HE Staining at 100X Magnification using a Trinocular Microscope.

structural support and provide nourishment, significantly impacts germ cells during spermatogenesis (Griswold, 2018). Furthermore, the death of Leydig cells which are the primary source of testosterone production as they are the essential hormone in the spermatogenesis process, can lead to hormonal imbalances (Zhao *et al*., 2019). Testosterone is transported to Sertoli cells via androgen-binding proteins, inducing and promoting spermatozoa maturation. Disruption of this hormone may result in a reduction in the average diameter and thickness of the seminiferous tubular germinal epithelium (Bahshwan *et al*., 2019). These findings align with Trindade *et al.* (2013), whose observations demonstrated that the diameter and germinal epithelial thickness of seminiferous tubules in untreated diabetic white rats were significantly lower compared to those in non-diabetic control rats.

K+ group which served as the positive control with metformin treatment at a dose of 50 mg/kg BW for 14 days, showed up results of average diameter of (258.58±19.67) and the germinal epithelium thickness was (76.32±9.07), in P1 group treated with 200 mg/kg BW of *R. tuberosa* leaf extract showed that the average diameter was (283.44±21.93), and the germinal epithelium thickness was (88.50±7.97), meanwhile in P2 group treated with 400 mg/kg BW of *R. tuberosa* leaf extract showed that the average diameter was (284.77±20.85) and the epithelium thickness was (86.54±7.47). All three groups showed normal morphological features, with the seminiferous tubule lumen containing many spermatozoa, and spermatogenic cells forming a clear boundary with the germinal cell cycle. The structural integrity of the tubules was also clearly observed. The P1 group showed a higher average epithelium thickness compared to P2, but the difference was not significant.

Plants containing antioxidants influence the regulation of hormones such as FSH, LH, and testosterone, which in turn can affect the growth and development of Sertoli cells and spermatogenic cells. A significant increase in the number of spermatogenic cells in the P1 and P2 groups compared to P0 group resulted in an increase in the diameter and thickness of the seminiferous tubule germinal epithelium. This is due to the antioxidant content of sorbifolin and pedalitin which can prevent oxidative stress and supporting spermatogenesis process (Asadi *et al*., 2017). The decrease in the diameter and thickness of the germinal epithelial cells in the P0 group resulted from the loss of Sertoli cells, inhibition of spermatogenic cells, and the potential occurrence of germinal cell apoptosis. Apoptosis can spontaneously occur in seminiferous tubules as a response to various environmental factors (Oyovwi *et al*., 2024). The study conducted by Trindade *et al.* (2013) showed a strong correlation between the duration of diabetes and the presence of tissue atrophy, highlighting that the duration of diabetes plays a role in the development of testicular lesions and the severity of diabetes.

The comparison of *R. tuberosa* leaf extract dosage treatments between group P1 and P2 in terms of diameter and thickness of the seminiferous tubule germinal epithelium did not show significant differences. In fact, the germinal epithelium thickness in group P2 (86.54±7.47) showed a decrease, with no significant difference compared to group P1 (88.50±7.97). This phenomenon is often observed with natural compounds, as the substances in these natural materials are not single compounds but rather consist of a variety of compounds that collectively produce effects. Therefore, the interaction between constituents is crucial (Caesar and Cech, 2019). The interactions among the compounds in the plant extract can result in synergistic, additive, or antagonistic activities, necessitating the use of the correct dosage to achieve both safety and efficacy of the extract (Olszowy-Tomczyk, 2020). This is consistent with the study by Baldim *et al.* (2017), which noted that when flavonoids level is high in *R. tuberosa* leaf extract, flavonoids can shift from an antioxidant role to a pro-oxidant one, leading to the loss of hydrogen atoms and subsequently generating free radicals in the body, causing cell damage. Research by Susetyarini *et al.* (2015) showed that a decrease in spermatogenic cell production could also be caused by alkaloid compounds, which can induce cell degradation and a reduction in spermatogenic cell count. Alkaloid compound can have cytotoxic effects, ultimately leading to a decrease in testosterone production.

Diabetes treatment currently uses metformin. This drug is derived from plants that is not specifically designed to target a particular disease pathway or mechanism (Triggle *et al*., 2022). Metformin is widely used to treat other conditions, including osteoporosis (Zhao *et al*., 2019), hemorrhoids (Tseng, 2021), inflammatory bowel disease (Wanchaitanawong *et al*., 2022), periodontitis (Najeeb *et al*., 2018), cancer (Mallik and Chowdhury, 2018), spinal cord injury, and even infertility (Rehman *et al*., 2018). Based on the data analysis for the groups treated with *R. tuberosa* leaf extract (P1 and P2) compared to the K+ group treated with metformin at a dose of 50 mg/kg BW, significant differences were observed. Both the P1 and P2 groups showed improvements in the germinal epithelium thickness and seminiferous tubules diameter compared to metformin. The results from the P1, P2, and K+ groups showed an increase in the average germinal epithelium thickness and seminiferous tubules diameter compared to the normal control and diabetic control groups. This suggests that both metformin and *R. tuberosa* leaf extract have similar effects in enhancing the germinal epithelium thickness and seminiferous tubules diameter.

Conclusion

Based on the results of the conducted study, it can be concluded that the administration of *R. tuberosa* can improve the germinal epithelium thickness and seminiferous tubules diameter in the alloxan-induced white rats. The administration of *R. tuberosa* leaf extract at doses of 200 mg/kg BW and 400 mg/kg BW are significantly affect the improvement of germinal epithelium thickness and seminiferous tubules diameter compared to the administration of 50 mg/kg BW of metformin.

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Conflict of interest

The authors have no conflict of interest to declare.

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