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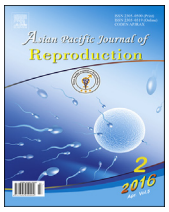

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Co-administration of caffeine and hydromethanolic fraction of *Citrullus lanatus* seeds improved testicular functions in alloxan-induced diabetic male Wistar rats

G.I. Onyeso[1], K.W. Nkpaa[2*], S. Omenihu[1]

[1]*Department of Physiology, Faculty of Basic Medical Sciences, College of Medicine, Madonna University, Elele, Rivers State, Nigeria*

[2]*Department of Biochemistry (Toxicology Unit), Faculty of Chemical Science, College of Natural and Applied Science, University of Port Harcourt, P.M.B 5323, Choba, Rivers State, Nigeria*

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ABSTRACT

Objective: To investigate the effect of *Citrillus lanatus* (*C. lanatus*) seeds and caffeine on blood glucose levels and testicular functions of alloxan-induced diabetic male Wistar rats.

Methods: Alloxan was administered at a single dose of 150 mg/kg BW to induce diabetes. A dose of either 200 mg/kg *C. lanatus* or 100 mg/kg caffeine or both was administered daily to alloxan-induced diabetic rats for three weeks, after which results were compared with a normal control group and a positive control group that received both alloxan and glybenclamide.

Results: *C. lanatus* seeds extract significantly decreases ($P < 0.05$) blood glucose level and significantly ($P < 0.05$) increased sperm motility, sperm count, normal sperm morphology, sperm viable cells and testosterone in plasma level of alloxan-induced diabetic rats treated with *C. lanatus* seed extract and caffeine.

Conclusions: The present study showed that co-administration of caffeine and hydromethanolic fraction of *C. lanatus* seed extract have hypoglycemic effect and may consequently ameliorate the impaired testicular general architecture and inhibits sperm death or testicular damage caused by alloxan-induced diabetes.

1. Introduction

According to the World Health Organization (WHO), there are approximately 160 000 diabetics worldwide, the number of diabetics has double in the last few years and is expected to double once again in the year 2025 [1]. Due to its high prevalence and potential deleterious effect on a patient physical and psychological state, diabetes is a chronic disorder caused by partial or complete insulin deficiency and it's a major medical concern [2].The disease remains incurable and can only be controlled with drugs. Despite the availability of medication for management of diabetes, the interest in alternative traditional remedies is increasing [3,4].

Citrullus lanatus (*C. lanatus*) (watermelon) seeds contains phytochemical constituents like alkaloids, flavonoids, tannins and Saponin with recognizable hypoglycemic effect as well as the presence of soluble fiber and carbohydrate [4–6]. *C. lanatus* helps in boosting antioxidant levels because it is exceptionally rich in carotenoids such as lycopene, lutein and β carotene [7]. A regular watermelon juice consumption result in significant increases in blood plasma concentrations of lycopene and β carotene [8–10].

A six weeks study found that treatment with a *C. lanatus* extract containing 6 g of L-citrulline and L-arginine daily on middle-aged obese subjects with prehypertension or stage one hypertension experienced reduced ankle blood pressure and altered carotid wave reflection, an indication of improved arterial function of the individuals [10–12]. It is very important to note that all parts of the watermelon have something to offer. For example, the seeds are excellent source of protein. The good nutritional and functional properties of watermelon seed meal proteins suggest their potential use in food formulations and diets [13]. *C. lanatus* possesses numerous bioactivities from natural source which is of better advantage than conventional therapies [14]. The study was carried out to investigate the effect of hydromethanolic fraction of *C. lanatus* seed extract

*Corresponding author: Nkpaa K.W., Department of Biochemistry (Toxicology Unit), Faculty of Chemical Science, College of Natural and Applied Science, University of Port Harcourt, P.M.B 5323, Choba, Rivers State, Nigeria.
Tel: +234 8066626323
E-mail: nkwilly@gmail.com
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and caffeine on blood glucose level and testicular functions of alloxan-induced diabetic male Wistar rats.

2. Materials and methods

2.1. Animal treatment

Thirty albino rats of male sex weighing between 200 and 250 g were used for this study. The animals were procured and then brought to the animal house of Madonna University Elele, kept in wooden cages and allowed to acclimatize for two weeks after which they were selected into six different groups according to their body weight with five in each groups (n = 5). Their health status was closely monitored in a clean wooden cage kept in a clear room. The cages were cleaned regularly to avoid infection of rats. These animals were fed with standard rat feed (Guinea feeds with composition: Protein 14.5%, fat 4.8%, fiber 7.2%, calcium 0.8%, phosphorus 0.62%, sodium 0.15% and metabolizable energy 2 300 kcal/kg, water *ad libitum*). The experimental protocol was approved by Institutional Animals Ethics Committee (IAEC), for using animals in this experiment. Animals were fasted overnight with free access to water prior to each experiment.

2.2. Extraction of watermelon seed

Ripe watermelon pods were obtained from the local market in Elele, rivers state, Nigeria. The seeds extracted from the pods after allowed rotting manually by washing, only healthy looking seeds [15], (brown in color, not floating on water, without mechanical damage or sign of infection) were collected. The collected seeds were oven-dried at 35 °C, until a constant weight was obtained. The dried seeds were reduced into powder using a laboratory grinding mail and frozen until used. The grinded seed was thereafter taken to the laboratory (Biochemistry laboratory of Madonna University), placed in a white bucket and weighed using an electric weighing balance. The weight of the bucket been 320 g, while the watermelon seed weighed as follows; 2 302, 526 and 570 g. Each of the measured watermelon seed was dissolved in 800 mL of methanol and 200 mL of water. These were divided in this manner for a better and accurate result. The buckets used for this processes were properly covered and kept for a 24 h period. After 24 h the different portions were combined and filtered using Whatman No.1 filter paper and funnel hung on a climb stand. The filtrate was collected in a glass jar as a golden colored liquid. The extract was thereafter placed in a water bath which made it concentrated to a gelatinous substance. After a week the concentrated hydromethanolic extract of *C. lanatus* seeds was dissolved in 2 litters of water and poured into a white bucket. Thereafter, the extract was then quantitatively transferred into amber colored bottles covered with aluminum foil and stored in a refrigerator at 4 °C before use. This is to prevent it from losing its potency. A dose of 200 mg/kg was used for high dose and 100 mg/kg for low dose administration as shown on Table 1. Alloxan (hydrate) LR, $C_4H_2N_2O_4 \cdot H_2O$ and glybenclamide ($C_{23}H_{28}ClN_3O_5S$) was purchased. Alloxan was dissolved in saline solution (0.9% sodium chloride, pH 7). The dose of alloxan used was 150 mg/kg as a single dose. This dose was chosen because it was effective to induce diabetes.

Treatments for the 6 groups were listed as following: Group I: Normal feed + water; group II: Normal feed + water + 100 mg/kg caffeine + 150 mg/kg Alloxan; group III: Normal feed + water + 150 mg/kg Alloxan + 5 mg/kg Glybenclamide; group IV: Normal feed + water + 200 mg/kg *C. lanatus* + 150 mg/kg Alloxan; group V: Normal feed + water + 150 mg/kg Alloxan; group VI: Normal feed + water + 100 mg/kg caffeine + 200 mg/kg *C. lanatus* + 150 mg/kg Alloxan.

2.3. Samples collection and analysis

After 30 days of treatment the animals were fasted for 24 h prior to sacrifice. The animals were anesthetized using chloroform and then sacrificed [10]. Thus, blood collected via cardiac punctured and put in a labeled Ethylenediaminetetraacetic acid bottle (EDTA) for testosterone hormonal assay and later centrifuged at 7 000 rpm for ten (10) minutes. The serum was then collected and stored at −15 °C. Hormonal assay was carried out on the sample a day after. The animals were then dissected; the testes were removed along with caudal epididymis. The caudal epididymis was separated from testes and lacerated to collect the semen with a microscope glass slide for analysis of sperm characteristics.

The blood glucose level was checked with a glucose strip and a glucometer. Initial glucose level before inducing alloxan was checked to ensure the rats were not already diabetic, after inducing alloxan the glucose level was checked as well which served as the initial glucose level before inducing and initial glucose level after inducing. The glucose level was checked weekly to see the effect of the extract on the glucose level of the rat.

2.4. Hormonal assay for testosterone

An enzyme-based immunoassay system was used to measure testosterone level in serum samples collected. Blood serum was introduced into micro – plate well for each sample to be measured. Thereafter, an enzyme antigen linked conjugate for testosterone was added to all and rocked for 10 s thereafter incubated for 1 h at room temperature. The plate was then

Table 1
Treatments groups used in this study.

Group	Treatments	Durations	Number of rats
I	Normal feed + water	21 days	5
II	Normal feed + water + 100 mg kg^{-1} CF + 150 mg kg^{-1} alloxan	21 days	5
III	Normal feed + water + 150 mg kg^{-1} alloxan + 5 mg kg^{-1} glybenclamide	21 days	5
IV	Normal feed + water + 200 mg kg^{-1} CL + 150 mg kg^{-1} alloxan	21 days	5
V	Normal feed + water + 150 mg kg^{-1} alloxan	21 days	5
VI	Normal feed + water + 100 mg kg^{-1} CF + 200 mg kg^{-1} CL + 150 mg kg^{-1} alloxan	21 days	5

washed with micro plate washer to remove all unbound material. After washing, excess fluids were taped off using dry paper towel. Then color was developed by adding color reagent to determine the bound hormone. Quantitative test result was obtained by measuring the absorbance. The color intensity was checked by tasking the ELISA plate to an ELISA reader which is attached to spectrophotometer which read the absorbance.

2.5. Histology of the testes

The testes were fixed in Formalin, after complete fixation the blocks was embedded in paraffin and sections cut at 5 µm (micron) using a microtome and then stained with hematoxylin and eosin and mounted in Canada balsam [16]. Microscopic examination of the sections was then carried out under a light microscope and later the microscopic slides of the testes were photographed at magnification 40×.

2.6. Statistical analysis

The result obtained from this study was analyzed using the statistical package for social science (SPSS) version 20.0 for windows. Analysis of variance (ANOVA) was used to compare means, and values were considered significant at $P < 0.05$. Post hoc multiple comparisons for differences between groups and within groups were established using least significant difference (LSD) turkey, Scheffe and Duncan.

3. Results

The results of the effect of co-administration of caffeine and *C. lanatus* seeds extract on blood glucose level of alloxan-induced diabetic male Wistar rats before and after induction are presented on Table 1. The blood glucose level of rats before alloxan induction in group IV (86.30 ± 2.56 mg/dL) significantly increase compared to group I and II (78.90 ± 7.70 and 63.20 ± 4.59 mg/dL). Although, after alloxan induction, the experimental groups II, III, IV, V and VI (357.5 ± 20.8, 308.70 ± 54.10, 290.80 ± 43.70, 235.80 ± 25.40 and 386.20 ± 25.30 mg/dL) significantly increased ($P < 0.05$) when compared with groups I (78.90 ± 7.70).

The results of the effect of the administration caffeine and *C. lanatus* seeds extract on weekly blood glucose level of alloxan-induced diabetic male Wistar rats are shown in Table 2. Group I showed no significant different ($P > 0.05$) across week 1, 2 and 3. Group II showed only a marginal increase across the three groups. While the group III significantly ($P < 0.05$) increased blood glucose level after a week (week I) from previous level shown in Table 1. The blood glucose level attained at week 1 in group III significantly ($P < 0.05$) decreased gradually in week 2 and 3. The group IV and VI showed a profound significant ($P < 0.05$) decrease in blood glucose level as shown in Table 2. While the blood glucose level in group V significantly ($p < 0.05$) increase across week 1, 2 and 3 (285.8 ± 38.4, 321.7 ± 35.3 and 389.3 ± 5.42 mg/dL) respectively (Table 2).

Results of the effects of acute administration of caffeine and hydromethanolic fraction of *C. lanatus* seeds extract on the sperm parameters and testosterone level and of alloxan-induced diabetic male Wistar rats are shown in Table 3. The result of the study on the sperm mobility between group II and III showed no significant difference, but there was a significant difference ($P < 0.05$) in sperm count between the two groups ($P < 0.05$). The percentage of normal sperm morphology of experimental rats in groups II, III, and V were significantly ($P < 0.05$) different from that of group I. The sperm viable cells in experimental animals groups II, III, and V were also significantly ($P < 0.05$) different from that of group I two. While, the testosterone level in groups III and V were significantly different ($P < 0.05$) when compared with group I.

Table 2
Blood glucose level before and after alloxan induction on male Wistar rats.

Groups	BGL before induction (mg dL^{-1})	BGL after induction (mg dL^{-1})
I	78.9 ± 7.70^{b}	78.9 ± 7.70^{a}
II	63.2 ± 4.59^{a}	357.5 ± 20.8^{b}
III	78.8 ± 6.47^{b}	308.7 ± 54.1^{c}
IV	86.3 ± 2.56^{c}	290.8 ± 43.7^{c}
V	76.3 ± 3.17^{b}	235.8 ± 25.4^{c}
VI	78.7 ± 4.01^{b}	386.2 ± 25.3^{b}

Data represented as Mean ± SEM; ^{a,b,c} within columns, between week 1–3, means with different superscripts letters differs significantly ($P < 0.05$).

Table 3
Showing the effects of co-administration of caffeine and hydromethanolic fraction of CL seeds extract on the weekly blood glucose level of alloxan-induced diabetic male Wistar rats.

Groups	Week 1 (mg dL^{-1})	Week 2 (mg dL^{-1})	Week 3 (mg dL^{-1})
I	69.5 ± 4.21^{a}	79.3 ± 2.29^{a}	72.5 ± 2.02^{a}
II	262.3 ± 20.3^{a}	266.8 ± 20.4^{a}	284.0 ± 32.9^{a}
III	237.5 ± 34.9^{a}	192.8 ± 54.3^{b}	128.8 ± 28.8^{c}
IV	250.3 ± 41.6^{a}	142.6 ± 23.5^{b}	96.5 ± 7.4^{c}
V	285.8 ± 38.4^{a}	321.7 ± 35.3^{b}	389.3 ± 5.42^{c}
VI	283.5 ± 40.5^{a}	141.9 ± 45.2^{b}	107.3 ± 4.96^{c}

Data represented as Mean ± SEM; ^{a,b,c} within row, between week 1–3, means with different superscripts letters differs significantly ($P < 0.05$).

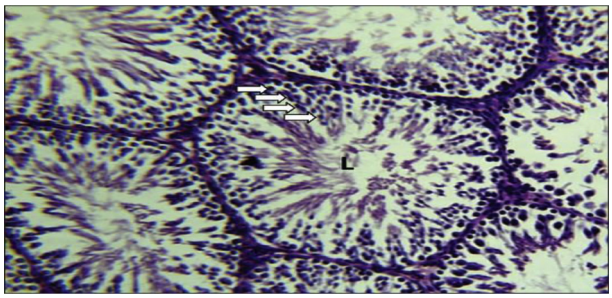

Figure 1. Photomicrograph of testis of experimental rats administered water (×200 H&E).

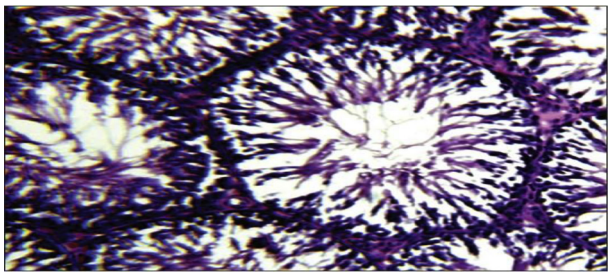

Figure 2. Photomicrograph of testis of experimental rats administered 100 mg/kg caffeine (×200 H&E).

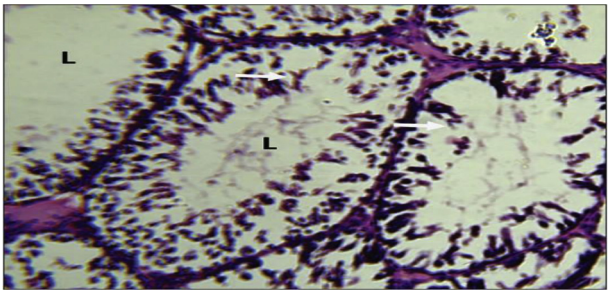

Figure 3. Photomicrograph of testis of experimental rats administered with150 mg/kg alloxan and 5 mg/kg glybenclamide (×200 H&E).

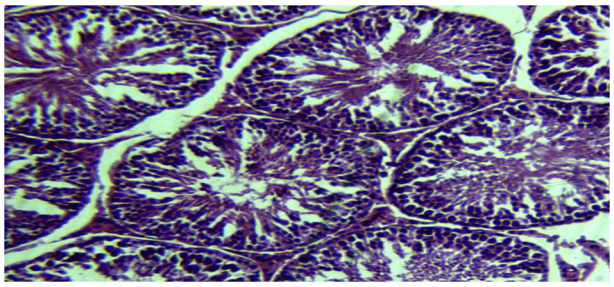

Figure 4. Photomicrograph of testis of experimental rats administered 200 mg/kg hydromethanolic fraction of *C. lanatus* seeds extract and 150 mg/kg alloxan (×200 H&E).

Results of the histological examination of the testis are shown in Figures 1–6. The testis of experimental rats in group I exhibited a normal testicular architecture and presence of several normal spermatocytes showing apparent normal outline of the seminiferous tubules, interstitium and spermatogenic cells (arrows). The spermatozoa are arranged in rows between and around the cells of sertoli. There was no thickening of the basement membrane. Tubules appeared healthy and no areas of fibrosis were found. Healthy quantities of Sertoli and Leydig cells were present as shown in Figure 1. Group II exhibited deeply stained microstructure and slight reduction in the population of spermatogenic cells. The interstitial spaces were wider than those of group I as shown in Figure 2. While testis in group III showed loss of spermatogenic cells, reduced density of mature spermatozoa within the lumens (L) of the seminiferous tubules, scanty supporting cells (arrows) and some degrees of disintegration and degeneration of the cells (Figure 3). However, histological sections of testis in group IV showed apparent normal testicular architecture as shown in Figure 4, while testis in group VI showed reduced staining intensity, wider luminal diameter, with reduced population of mature sperm cells in the lumen (Figure 6). Moreso, testis in group V showed atrophy and degeneration of seminiferous tubules and loss of spermatogenesis, decrease in spermatogenic cell layer and germinal epithelium (Figure 5).

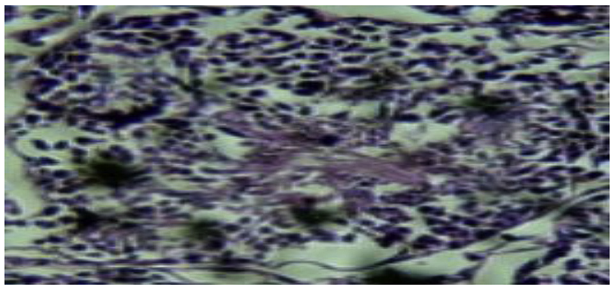

Figure 5. Photomicrograph of testis of experimental rats administered 150 mg/kg alloxan (×200 H&E).

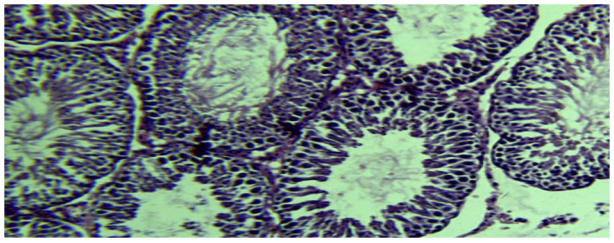

Figure 6. Photomicrograph of testis of rats administered 100 mg/kg caffeine, 200 mg/kg hydromethanolic fraction of *C. lanatus* seeds extract and 150 mg/kg alloxan (×200 H&E).

4. Discussion

Alloxan has been observed to cause a massive reduction of the B-cells of the islets of Langerhans and induce hyperglycemia [17,18]. In our study we have found that co-administration of caffeine and hydromethanolic fraction of *C. lanatus* seeds extract decreases blood glucose in alloxan diabetic rats, which is consistent with study done by Omigie and Agoreyo [4]. Also, administration of glybenclamide (a known hypoglycemic drug) significant decrease blood glucose level. However administration of *C. lanatus* seeds extract only exhibited far more hypoglycemic effect when compared with co-administration of caffeine and hydromethanolic fraction of *C. lanatus* seeds extract and glybenclamide alone. Nasiri *et al.* [19] also reported that *C. lanatus* seed have hypoglycemic effect. The possible mechanism by which administration of hydromethanolic fraction of *C. lanatus* seeds extract brings about its hypoglycemic action may be by potentiating the insulin effect by increasing either the pancreatic secretion of insulin from the B-cells of islets of Langerhans or its release from bound insulin. This result is in consistent with Mahesh and Menon, [20] study which reported that the effect of flavonoid which is one phytochemical of *C. lanatus* on pancreatic B-cells may lead to proliferation and secretion of more insulin. It could also be that the extract caused a hypoglycemic effect by inhibiting the process of glycogenolysis (break down of glycogen to form glucose) or it inhibited the process of glycogenesis by the liver.

The diabetic hyperglycemia induces reduction of sperm parameters and testosterone level which are considered to be significant biomarkers of male fertility. This study showed significant decrease in sperm motility, sperm count, normal sperm morphology, sperm viable cells and testosterone in diabetic groups. These results indicated that diabetes could lead to impotence in male. However, after treatment of with caffeine and hydromethanolic fraction of *C. lanatus* seeds extract, the level of sperm motility, sperm count; normal sperm morphology, sperm viable cells and testosterone in alloxan-diabetic groups significantly increased. This could be due to the presence of a phytonutrient citrulline, found in watermelon seed. When it is consumed citrulline has an added benefit, it is converted to arginine which has been known to boost sperm count in men [21]. Also, arginine is an important amino acid also found in caffeine. Nasir [5] reported that *C. lanatus* contains tannins and saponin with recognizable hypoglycemic effect as well as the presence of soluble fiber and carbohydrate which may contribute to the hypoglycemic effect.

The health benefit of *C. lanatus* may also be attributed to the presence of phenol and flavonoids in watermelon seed and

Table 4
Effects of Acute Administration of caffeine and hydromethanolic fraction of CL seeds extract on the sperm parameters and testosterone level and of alloxan-induced diabetic male Wistar rats.

Groups	Sperm motility (%)	Sperm count ($\times 10^6$ mL^{-1})	Normal sperm morphology (%)	Sperm viable cells (%)	Testosterone (ng/ml)
I	82.3 ± 2.33^a	67.0 ± 1.15^a	82.7 ± 1.45^a	90.0 ± 1.15^a	3.61 ± 0.34^a
II	51.3 ± 1.76^b	52.3 ± 3.38^a	73.3 ± 3.48^b	80.8 ± 1.15^b	2.53 ± 0.14^a
III	54.3 ± 2.33^b	24.6 ± 4.05^b	38.3 ± 3.75^c	56.1 ± 3.22^c	1.99 ± 0.37^b
IV	82.0 ± 1.15^a	65.7 ± 1.45^a	79.3 ± 0.88^a	87.3 ± 0.88^a	3.59 ± 0.24^a
V	35.0 ± 2.08^c	22.8 ± 1.76^b	28.6 ± 1.76^c	50.7 ± 1.85^c	0.31 ± 1.17^c
VI	78.7 ± 2.03^a	62.3 ± 1.76^a	77.3 ± 2.60^a	86.5 ± 0.88^a	4.52 ± 0.34^a

Data represented as Mean ± SEM; ^{a,b,c} within columns, between control and treated animals, means with different superscripts letters differs significantly ($P < 0.05$).

caffeine [22]. The property of almost every flavonoid is their capacity to act as an antioxidant [22]. Therefore the high phenolic and flavonoid content of watermelon seed and caffeine suggest possible high antioxidant potential that helps to return the sperm motility towards normal. Also, the significant increase seen in group VI on Table 4 may be due to the presence of ascorbic acid and flavonoid in caffeine and watermelon seed reported by Edward *et al.*, [8] which is known to influence the synthesis of luteinizing hormone from the anterior pituitary gonadal axis. Luteinizing hormone stimulates the leydig cells in the production of testosterone [23].

The loss of spermatogenic cells, reduced density of mature spermatozoa within the lumens (L) of the seminiferous tubules, scanty supporting cells (arrows), disintegration and degeneration of the cells indicated that diabetes may induce sperm death. Also, alloxan-induced diabetes lead to atrophy and degeneration of seminiferous tubules and loss of spermatogenesis decrease in spermatogenic cell layer and germinal epithelium. This also resulted in shrunken seminiferous tubules and reduced cellularity. The blood vessels were also noted to be dilated and congested. All these suggested that the testis was heavily damaged by alloxan administration as shown in Figure 5. On the other hand, treatment of the diabetic rats with caffeine and *C. lanatus* normalized the general architecture of the testis. From the results above it could be concluded that co-administration of caffeine and hydromethanolic fraction of *C. lanatus* seeds extract are able to normalize the blood glucose levels and could ameliorate the impaired testicular general architecture and inhibit sperm death.

Conflict of interest statement

The authors declare that there is no conflict of interests.

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