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Original Research

Protective Effect of *Vitis gracilis* Wall (Vitaceae) Leaf Decoction on Sexual Vitality and Testis of Alloxan-Induced Diabetic Mice

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Abstract

Diabetes mellitus is profoundly associated with various detrimental outcomes including sexual dysfunction and infertility in males. On the other hand, a medicinal plant namely *Vitis gracilis* Wall (Vitaceae) has been used as a traditional medicine to enhance vitality. This present study aimed to investigate the protective effect of *V. gracilis* leaf decoction against diabetes-induced sexual dysfunction and testicle histopathology in adult male mice. The experiment was composed of five different groups namely the control (non-diabetic) group, the diabetes group (without any treatments), and the diabetes treated with *V. gracilis* decoction at the doses of 25, 50, and 100 g/L, respectively. In addition, the phytochemical constituents of leaf decoction were determined by using Ultra performance-liquid chromatography-mass spectroscopy (UPLC-MS). Our data demonstrated that, despite failing to improve blood glucose profile and body weight, *V. gracilis* leaf decoction sustained intense sexual behaviors including face and genital kissing, genital licking, and mount toward estrous females. Moreover, lower doses of decoction (25 and 50 g/L) attenuated the diabetes-induced reduction of testis weight and precluded malondialdehyde accumulation in the testicle tissue. The decoction at the lower doses also ameliorated histopathological alterations in the testis, particularly the wall thickness of tubulus seminiferous and the number of necrotic cells. *V. gracilis* decoction also improved hematological values including hemoglobin, red blood cell count and hematocrit level. In addition, UPLC-MS analysis revealed a total of 26 phytochemical compounds with seven predominant substances. In conclusion, leaf decoction of *V. gracilis*, particularly at lower doses but not at a higher dose, exerted a protective effect on sexual vitality, testicle tissue, and hematological value under diabetic condition. The beneficial effects of *V. gracilis* decoction might be associated with its various bioactive compounds. Therefore, *V. gracilis* leaves may be a future candidate as a potent natural drug for male sexual vitality and testicle protection against diabetes.

Keywords: Diabetes mellitus; Male fertility; Malondialdehyde; Sexual dysfunction; Vitaceae

Introduction

Diabetes mellitus is profoundly associated with various detrimental outcomes including sexual dysfunction and infertility in males [1]. A previous study indicated that the prevalence of erectile dysfunction in diabetic patients was markedly high, particularly those with old age, longer diabetes duration, and poor glycemic control [2]. A similar finding was also reported in type 2 diabetes patients [3]. Moreover, sexual dysfunction in people with type 1 diabetes has been indicated to be closely correlated with psychological problems including stress thereby reducing their quality of life [4]. However, the utilization of synthetic drugs to retain sexual functions in diabetic patients could lead to serious unwanted side effects. It is suggested that some prominent drugs for erectile dysfunction such as sildenafil, tadalafil, and vardenafil could increase the risk of cardiac toxicity [5].

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Hence, explorative studies focused on natural-based medicines to counteract diabetes-induced sexual dysfunction are urgently needed.

Plants in the genus of *Vitis* (Family Vitaceae) have been suggested to exert several medicinal benefits against various diseases including sexual dysfunction and metabolic disorders. For instance, a previous study reported that nutraceuticals containing *V. vinifera* extract could improve erectile dysfunction in patients with type 2 diabetes mellitus [6]. An *in vitro* study also revealed that *V. vinifera* extract effectively prevented oxidative stress-induced cell death [7]. The protective effect of *V. vinifera* is attributed to its high level of antioxidants [8]. Another investigation found that the extract of *V. vinifera* upregulated the expression of genes encoding endogenous antioxidant enzymes including catalase, superoxide dismutase, and glutamate transferase. Moreover, another study demonstrated the inhibitory effect of *V. vinifera* against pancreatic lipase activity thereby reducing the risk of obesity and associated disorders [9]. It has been also reported that the extracts, fractions, and purified compounds from *V. thunbergii* var. taiwaniana could effectively mitigate adiposity and dyslipidemia in high-fat diet-fed mice [10]. A report indicated that a compound namely (+)-ε-viniferin from *V. thunbergii* var. *taiwaniana* exerted anti-adiposity *in vitro* while lowering blood glucose levels in vivo in mice fed a high-fat diet [11].

Another wild species of Vitaceae namely *V. gracilis* Wall, locally known as "Gagatan harimau" in Indonesia, is suggested as a potent medicinal plant. The decoction of *V. gracilis* leaf is commonly used in traditional practices by Karonese people (a local tribe in North Sumatra, Indonesia) and is believed to enhance vitality. Some groups of bioactive compounds have been detected in the leaf extract of *V. gracilis* including terpenoids [12], alkaloids, glycosides, flavonoids, saponins, and tannins [13]. An experimental study using male mice revealed that the ethanolic leaf extract of *V. gracilis* profoundly exerted a protective effect on gastrocnemius muscle cells under a high-intense swim exercise by reducing inflammatory response and cytochrome c expression [13]. Although some medicinal benefits of *V. gracilis* have been scientifically indicated, the beneficial effect of this plant species against diabetes-induced sexual dysfunction remains unraveled. Moreover, the particular phytochemical constituents of the plant remain less elucidated. Hence, our present study aimed to investigate the protective effect of *V. gracilis* leaf decoction against sexual dysfunction and testicle histopathology in alloxan-induced diabetic mice as an animal model. We hypothesized that *V. gracilis* leaf decoction is effective in sustaining sexual functions against diabetes.

Materials and Methods

Sample collection and preparation of leaf decoction

Fresh samples of *V. gracilis* were provided by a local villager in Langkat, North Sumatra Indonesia on October 2022 and taxonomically validated by a certified botanist in the Biology Department, Faculty of Mathematics and Natural Sciences, Andalas University, Indonesia (voucher specimen deposit number: 1783/Bio/Unand). The leaf decoction and the dosages were prepared as per the protocol described previously [14]. Briefly, 1000 g of fresh leaves were washed five times with distilled water and subsequently sliced to be small pieces before being shade dried for 5-6 days at room temperature to achieve a constant weight. Afterward, the leaves were divided into three different portions such as 25 g, 50 g and 100 g then soaked into 1000 mL distilled water and boiled at 100 °C for 20 minutes in a water bath (TSGP20, Thermo Scientific, Massachusetts, USA), respectively. Thereafter, it was filtered using filter paper, cooled to room temperature and stored in an isolated dark bottle until being used in the experiment.

Phytochemical analysis of V. gracilis leaf decoction

Phytochemical compounds of *V. gracilis* decoction were identified by using UPLC-MS (ACQUITY UPLC®H-Class System equipped with LC column HSS C18; Waters, USA). The LC was set as follows: column temperature 50 °C, the mobile phase was composed of distilled water combined with 5 mM ammonium formic (A) and acetonitrile combined with 0.05% formic acid (B), the flow rate was set at 0.2 mL/min (step gradient) run for 23 min. The setting for MS (Xevo G2-S QT of, waters, USA; with electrospray ionization) is as follows: positive mode mass analysis range 50-1200 m/z, source temperature 100 °C, desolvation temperature 350 °C with cone gas flow 0 L/h, desolvation gas flow 793 L/h, collision energy 4 volts (low energy), ramp collision energy 25-60 volt (high energy). The decoction sample was first dissolved in ultrapure water (1 g/2 mL) followed by a brief centrifugation. Thereafter, 5 µL of supernatant was injected into the LC column and the analysis was run for 23 min. Subsequently, the results were analyzed by using Masslynx v4.1 software (Waters, USA).

Provision of animals

Thirty adult mice composed of 25 males and 5 females (BALB/c strain, 2 months old, 24-25 g of body weight) were provided by Pondok Tikus (Padang, West Sumatra). Upon arrival in the lab, mice were reared in an individual cage (one mouse per cage) in an animal room under regulated temperature and humidity (25

°C, 67%) and light/dark cycle (12 h dark/12 h light). During seven days of acclimatization, mice were fed with a standard rodent chow diet (Ratbio; Citra Inna Feed, Jakarta) and tap water ad libitum. The 25 male mice were assigned for experimental treatments, while the five females were prepared for sexual behavioral tests towards experimentally treated male mice. All protocols for animal handling and uses in this study were accordance with the standard guidelines for animal care and use of institutional research ethic committee of Andalas University (approval No.5210/UN-AND/2022).

Diabetic induction and experimental treatments

At the end of acclimatization, 20 mice were randomly chosen to be subjected to diabetic induction using alloxan monohydrate (Sigma Aldrich, Germany); while the other 5 mice were assigned as control (healthy non-diabetic group) without diabetic induction. The induction was performed by intraperitoneal injection of a single dose of alloxan (150 mg/kg of body weight; BW) [15]. Thereafter, blood glucose was monitored three days later using a glucometer (Dr Glucose AGM-2100, South Korea). The animal was justified as diabetic if the blood glucose level was 250 mg/dL or higher. Accordingly, all 20 mice injected with alloxan were confirmed as diabetic; thus, all individuals were included in the experiment. Furthermore, the 20 individuals of diabetic mice and 5 non-diabetic mice were assigned to five different groups (n=5 per group) according to the experimental design namely non-diabetic mice (non-diabetes mellitus; non-DM), alloxan-induced diabetic mice (DM), alloxan-induced diabetic mice + decoction 25 g/L (DM + 25 g/L of *V. gracilis*; Vgs), alloxan-induced diabetic mice + decoction 50 g/L (DM + 50 g/L Vgs), and alloxan-induced diabetic mice + decoction 100 g/L (DM + 100 g/L Vgs). The treatments were immediately started on the day of diabetic status justification and continuously carried out for 28 days. *V. gracilis* leaf decoction was given orally once a day (0.5 mL/ individual) in the morning (09.00); while the others (non-DM and DM) were given distilled water (0.5 mL/ individual).

Body weight and blood glucose measurements

Body weight was determined at the beginning and at the end of the treatments. Mice were weighted using a digital balance (Ohaus NVT2201, Ohaus New Jersey, USA). Blood glucose levels were also monitored at the beginning and the end of treatments. The blood sample was drawn from the tail vein gently and then applied to a glucometer to examine its blood glucose value. Both body weight and blood glucose measurements were carried out in the morning (08.00-09.00).

Sexual activity tests

At the end of treatments, mice were subjected to behavioral tests toward estrous females. Before the test, the estrous state of the females was determined by means vaginal swab (Byers *et al*., 2012). Then, each tested male mouse was paired with a designated female in a cage monitored with a camera (Olympus Tough TG6 Digital Camera, Olympus Tokyo, Japan). During the 10 min of pairing, the sexual activities of each male toward the female including face kissing, genital kissing, genital licking, and mounting were recorded and subsequently analyzed. Other activities were excluded from the analysis. The incidence of each type of sexual activity was presented as a percentage for each group of treatment. All the tests were carried out in a day (09:00-10:30). After the tests, animals were returned to their cages.

Measurement of hematological values

Two days after behavioral tests, mice were sacrificed using dislocation of vertebrae cervicalis. The blood sample was immediately collected from the cardiac puncture and transferred to a vacutainer containing EDTA. Next, hematological values including Hb concentration, erythrocyte count, and HTC were measured using an automated hematology analyzer (MINDRAY BC-2130, ICEN Technology Co Ltd., China).

Testicular sampling and observations

After blood sampling, the testicles were collected and weighed. The left side testicle was cut into two parts equally. A part was transferred into a microtube and kept at -80 °C for MDA measurement, while another part was fixed in the formalin 10% for histopathological examination.

Malondialdehyde (MDA) measurement

The MDA measurement in the testicle tissue was performed using a lipid peroxidation (MDA) assay kit (colorimetric/fluorometric) (Ab118970, Abcam, USA) following the previously described procedure [16]. The sample absorbances were determined by a spectrophotometer (SmartSpecTM Plus BioRad, USA).

Histopathological examination

The histological slides of testicles were prepared as per the protocol described by a previous report [17] and stained with hematoxylin-eosin (HE). The microscopic observations were performed using a light microscope (Olympus CX 33, Tokyo, Japan) equipped with a camera beta 3.1b MB (Sony Exmor, Sony Tokyo, Japan) on five slices of each mouse and were examined with four field views for each slice. The pictures were analyzed using Image J software (National Institutes of Health, USA) to quantify the number, di-

ameter and wall thickness of seminiferous tubules as well as the number of necrotic cells.

Data analysis

Data are presented as mean ± SE. The quantitative data were subjected to variance analysis followed by the Bonferroni post hoc test with $P<0.05$ was set as significant using IBM SPSS Statistics Base 22.0 for Windows.

Results

Effect of V. gracilis leaf decoction on body weight and blood glucose

After 28 days of treatment, final body weight significantly increased as compared with initial weight ($P<0.05$) in healthy mice (non-diabetic group) (Figure 1A). However, an alloxan-induced diabetic condition caused an apparent reduction in body weight increase, indicating a detrimental effect of diabetes on body weight. Among treatment groups, only mice treated with a lower dose of *V. gracilis* (25 g/L) exhibited substantial elevation in body weight ($P<0.05$). Otherwise, higher doses of decoction (50 and 100 g/L) failed to promote marked body weight gain. The body weight increase in the lower-dose treated group was also incomparable to the non-diabetic group ($P<0.05$). In three days after alloxan injection, blood glucose markedly raised in mice and became substantially different as compared with the non-diabetic group (Figure 1B). However, blood glucose levels also remained higher (>400 mg/dL) in diabetic groups including those treated with *V. gracilis* decoction (Figure 1B), indicating a lack of effectivity of *V. gracilis* in alleviating the diabetic condition. There was a tendency that final blood glucose decreased in mice treated with 50 and 100 g/L of decoction. Unfortunately, such reduction was statistically insignificant as compared with other treated groups.

Effect of V. gracilis leaf decoction on sexual activity

Behavioral observation performed at the end of treatment (Figure 2) demonstrated a deleterious effect of diabetes condition on sexual activities. Non-treated diabetic mice exhibited an apparent reduction in all parameters of sexual activity tests, including the incidence of face kissing (66.7%), genital kissing (66.7%), genital licking (16.7%), and mounting (0%) towards estrous mice. However, the treatments with various doses of *V. gracilis* leaf decoction sustained intense sexual activities that nearly became comparable to non-diabetic mice. Incidence of face kissing ranged from 83.3-100%, genital kissing was 100%, genital licking ranged from 33.3-80.0% and mounting ranged from 33.3-40% in *V. gracilis* decoction-treated groups. The incidence of mounting was even higher (1.9 to 2.4 times) in decoction-treated groups as compared with the non-diabetic group.

Effect of V. gracilis decoction on testis

The measurement of testis mass (Figure 3A) found a marked reduction in testis weight in alloxan-induced diabetic mice (DM) as compared with the non-DM

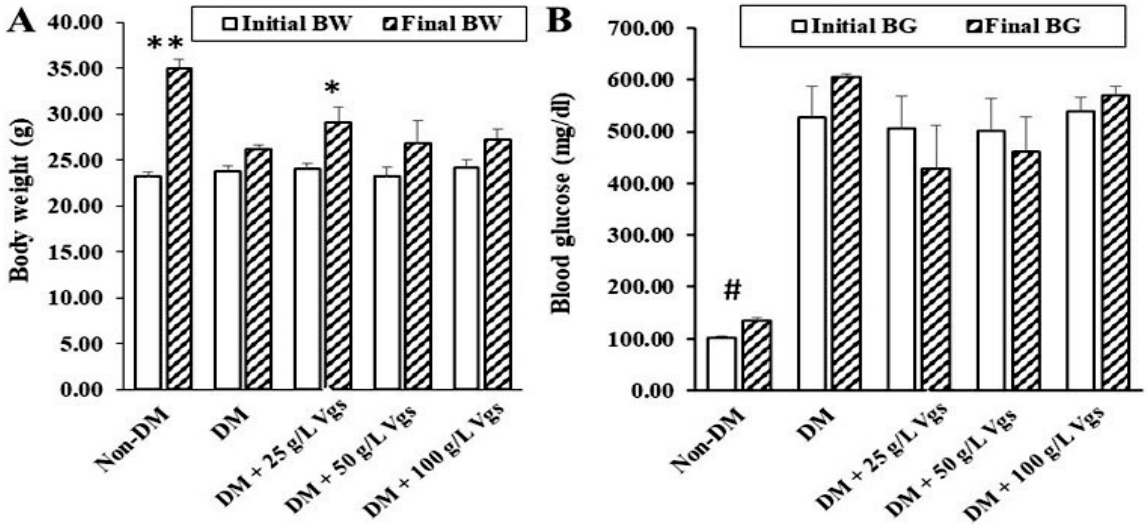

Figure 1. Effect of *V. gracilis* leaf decoction on body weight and blood glucose of alloxan-induced diabetic mice. (A) Body weight measured at the beginning and the end of the treatment, (B) random blood glucose determined at the beginning and the end of treatment. ** and * indicate statistical significance between initial and final body weight with $P<0.01$ and $P<0.05$, respectively, while # indicates a significant difference between the non-DM group with all other groups ($P<0.01$). n = 5 for each group.

group (P<0.05). However, in decoction-treated mice, particularly at the doses of 25 g/L and 50 g/L, testis weight was higher and became comparable statistically with non-DM group (P>0.05). Higher dose of *V. gracilis* leaf decoction caused a marked reduction in testis weight to be comparable with those in the diabetic group without any treatments (P>0.05). Furthermore, the determination of MDA level in testicle tissues (Figure 3B) revealed a noticeable elevation of MDA in the diabetic group as compared with other groups (including non-DM, 25 g/L and 50 g/L-treated groups; P<0.05). There was no significant difference in

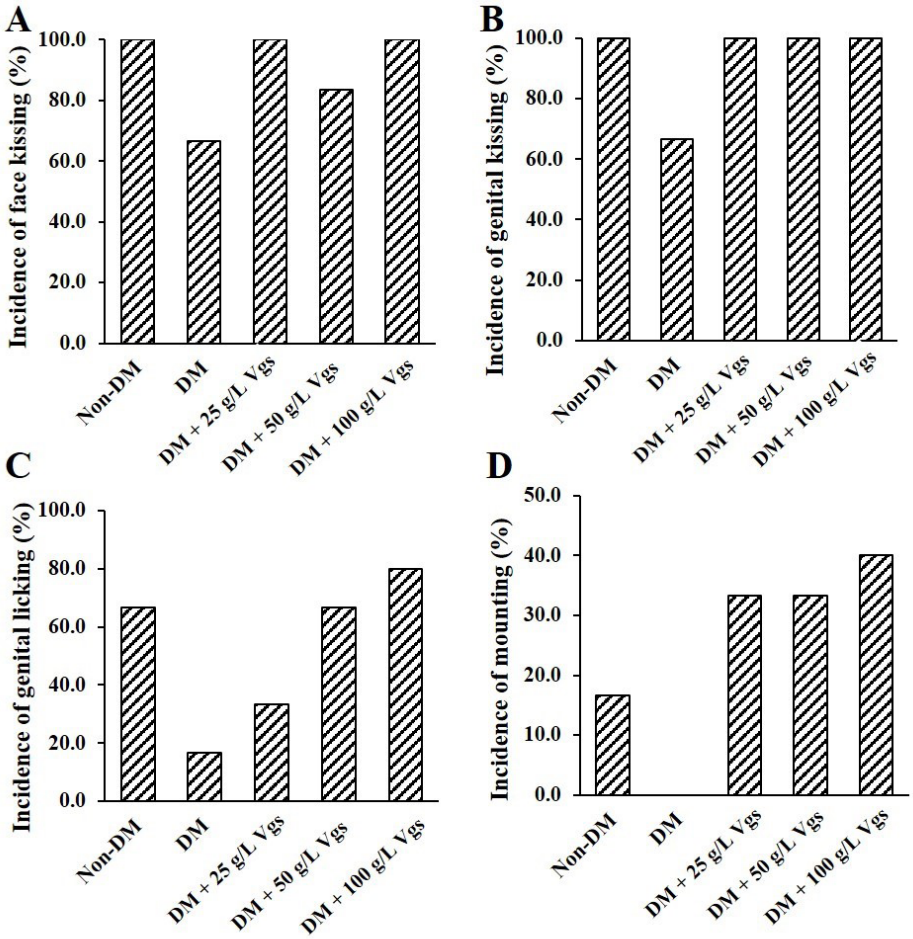

Figure 2. Effect of *V. gracilis* leaf decoction on sexual activities toward estrous female. (A) percentage of individuals exhibited face kissing, (B) genital kissing, (C) genital licking, and (D) mounting toward estrous females. n = 5 for each group.

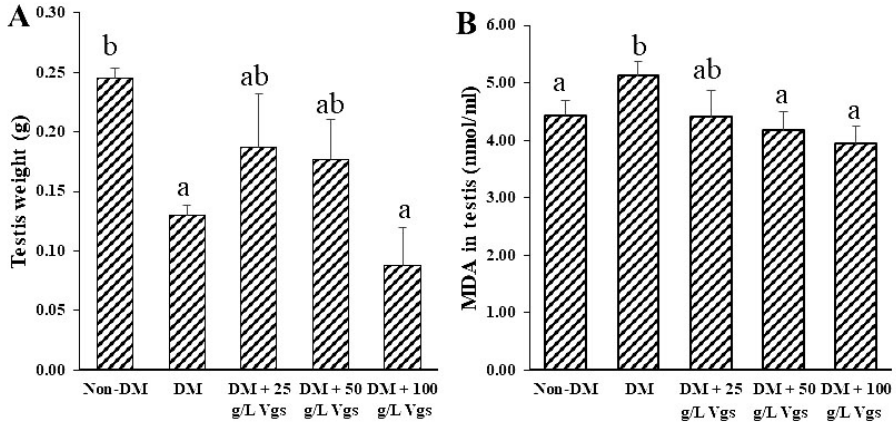

Figure 3. Effect of *V. gracilis* leaf decoction on testicle weight and MDA level in testicle tissues. Different lower-case characters above the bars represent statistical significance (P<0.05) with particular notes as follows: a (not significantly different with a), b (significantly different with a), ab (not significantly different with a and b).

MDA level among all decoction-treated groups (P>0.05). Thereafter, macroscopic observation of the testis indicated no noticeable difference in testis morphology among all groups of treatments, except for their smaller size of testis in those treated with the highest dose of *V. gracilis* decoction (100 g/L) (Figure 4, left panels). However, a microscopic examination (Figure 4, middle and right panels) revealed severe histopathological alterations in alloxan-induced diabetic mice as compared with the non-diabetic group. Under the non-diabetic condition, tubulus seminiferous were intact with a clearly visible wall, lumens and spermatozoa. In contrast, the testis of mice in the diabetic group (without decoction treatments) exhibited apparent destruction of tubulus seminiferous, disintegrated wall, and lack of complete structure of spermatozoa. These pathological alterations were attenuated in mice treated with 25 and 50 g/L of *V. gracilis* leaf decoction. However, at a higher dose (100 g/L), decoction apparently promoted structural damage in the testis.

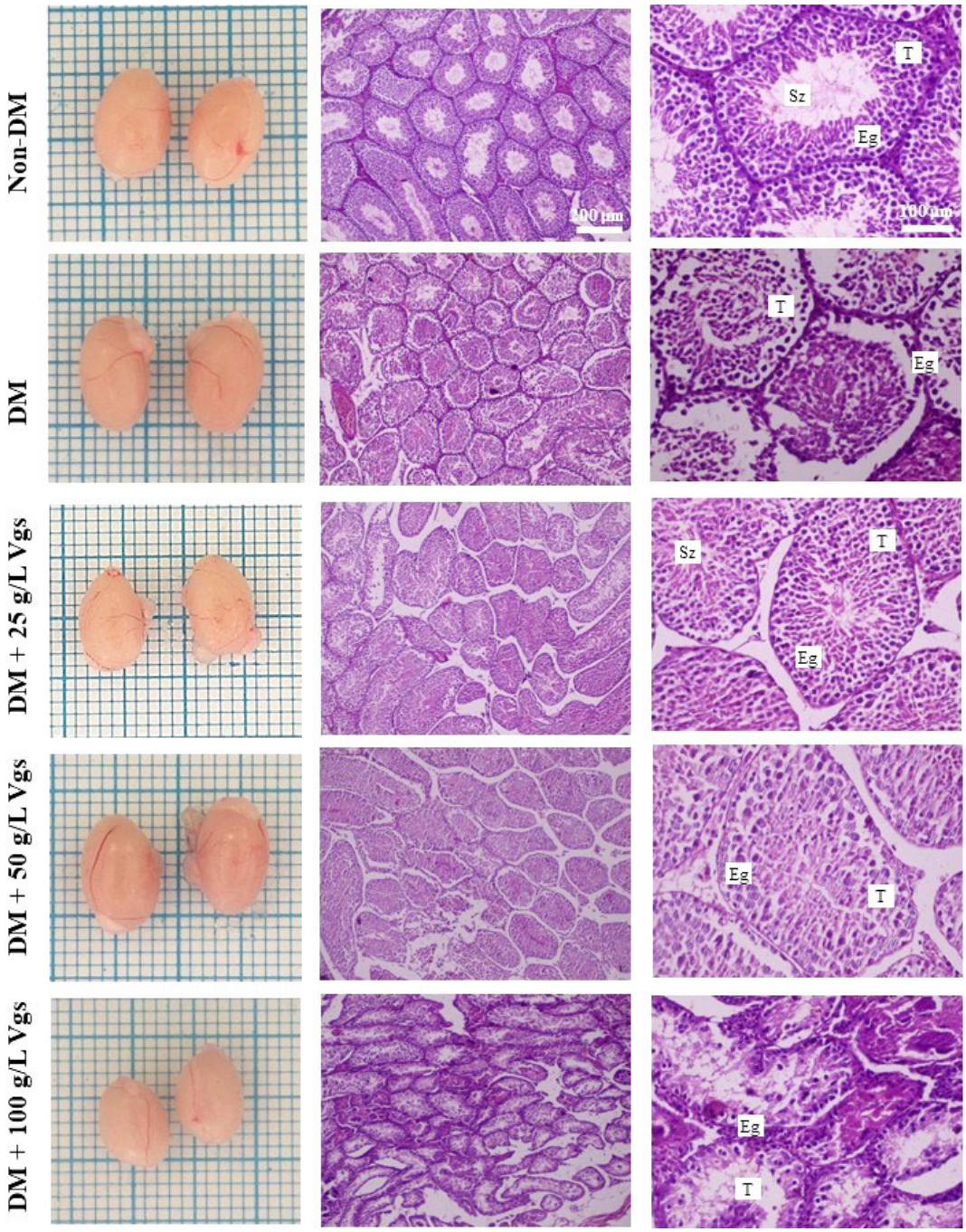

Figure 4. Effect of *V. gracilis* leaf decoction on morphology and histopathology of the testis. The left panels are testis morphology, middle panels and right panels are microphotographs of testis at a lower and higher magnification, respectively. Tissues were stained with HE. T (tubulus seminiferous), Sz (spermatozoa), Eg (epithelial germinativum).

Histomorphometric analysis on testis (Figure 5) found that diabetic condition caused a reduction in seminiferous tubule size, indicated by a significant increase in the number of observed seminiferous tubules per field view (Figure 5A) in the DM group as compared with non-DM (P<0.05). Interestingly, the tubulus seminiferous number was also lower in groups treated with *V. gracilis* leaf decoction at the doses of 25 and 50 g/L. Otherwise, the highest dose of decoction (100 g/L) exhibited a markedly high number of seminiferous tubules per field view and became comparable with those in the DM group (P>0.05). The diameter of seminiferous tubules (Figure 5B) was substantially smaller in DM and all decoction-treated groups as compared with the non-DM group (P<0.05). However, mice treated with the highest dose of decoction (100 g/L) depicted the smallest diameter of seminiferous tubules which was incomparable to other groups (P<0.05). The thickness of tubules (Figure 5C) markedly decreased in the DM group as compared with a non-DM group and those treated with decoction at the doses of 25 and 50 g/L (P<0.05). However, it was comparable to mice treated with *V. gracilis* decoction at the dose of 100 g/L (P>0.05).

Further examination revealed that the number of necrotic cells was markedly elevated in the testis of mice under the diabetic condition without any treatments compared to those in the non-DM group (66.6 times increase; P<0.01). On the other hand, the necrotic cells were substantially lower in mice treated with 25 and 50 g/L of decoction (P<0.05), but remained higher

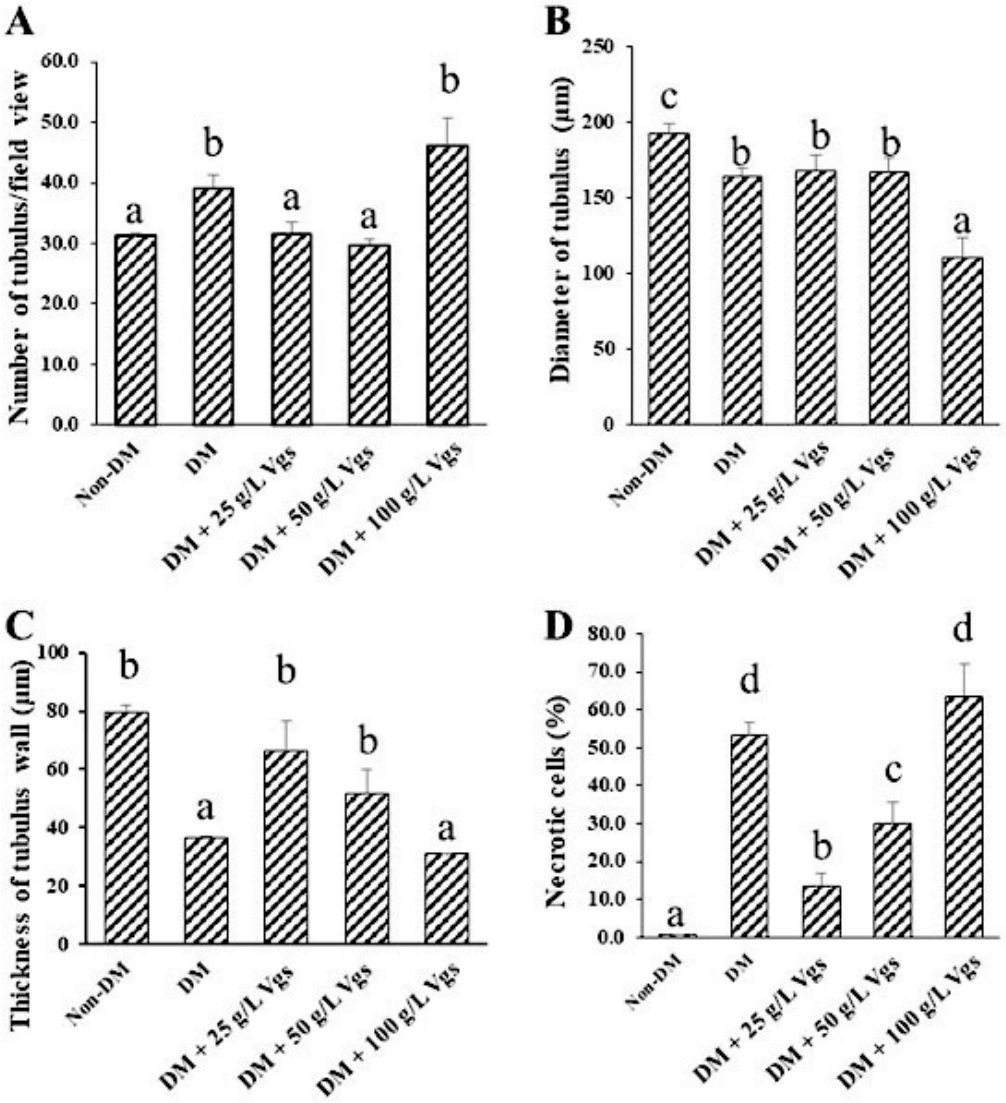

Figure 5. Effect of *V. gracilis* leaf decoction on histomorphometry of testicle. (A) number of seminiferous tubules per field view, (B) diameter, (C) wall thickness of seminiferous tubules, (D) percentage of necrotic cells in the testicle. Different lower-case characters above the bars represent statistical significance (P<0.05) with particular notes as follows: a (not significantly different with a), b (significantly different with a), c (significantly different with a and b), d (significantly different with a, b, and c) based on Bonferroni post hoc test.

in those treated with 100 g/L of decoction (P>0.05), as compared with the DM group.

Effect of V. gracilis leaf decoction on hematological values

In addition to sexual vitality and testicular histopathology, hematological values were also assessed. As depicted in Figure 6, the diabetes condition induced a significant reduction in Hb (Figure 6A), red blood cell (RBC) count (Figure 6B) and hematocrit (HTC) (Figure 6C), as compared with the non-DM group (P<0.05). Otherwise, *V. gracilis* leaf decoction, particularly at the dose of 25 g/L could sustain the Hb, red blood cell count and HTC value to be comparable with those in the non-DM group (P >0.05). However, such positive effects were not observable in mice treated

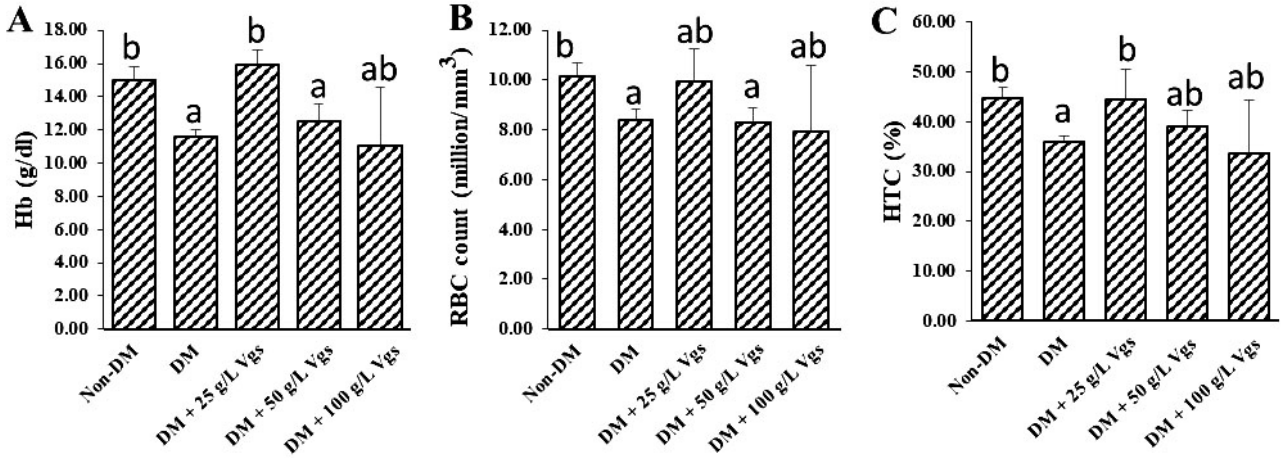

Figure 6. Effect of *V. gracilis* leaf decoction on hematological values. (A) Hb concentration, (B) number of red blood cells, (C) HTC value. Different lower-case characters above the bars represent statistical significance (P<0.05) with particular notes as follows: a (not significantly different with a), b (significantly different with a), ab (not significantly different with a and b) based on Bonferroni post hoc test.

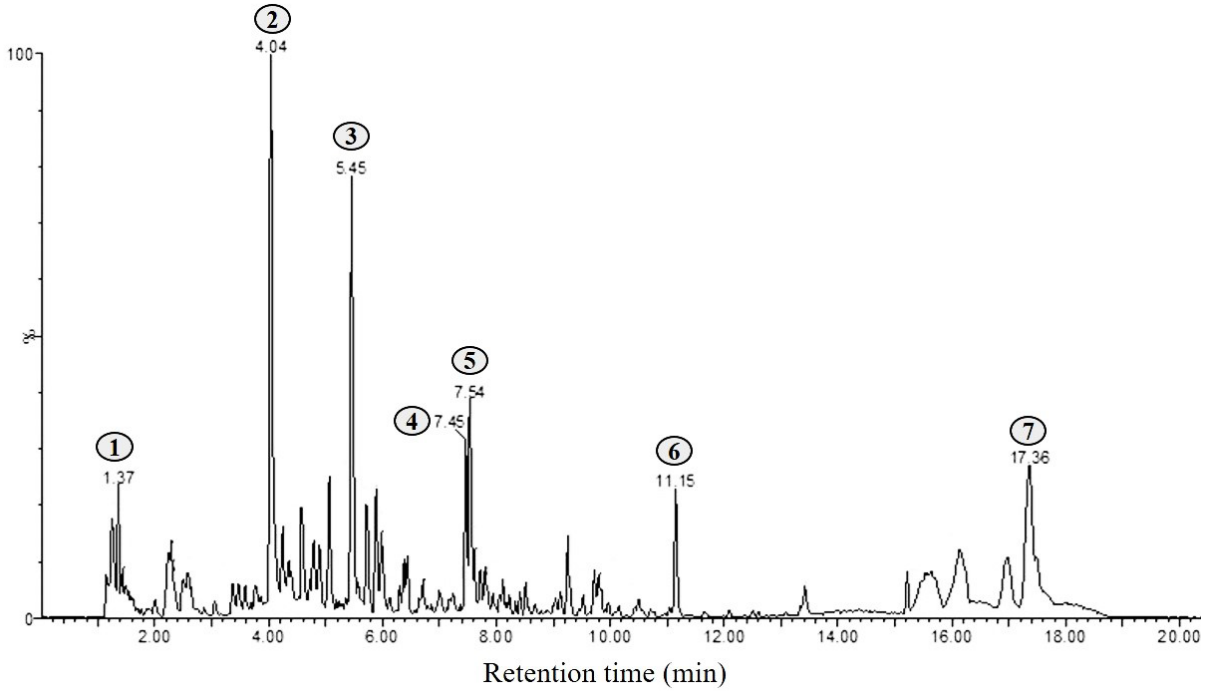

Figure 7. UPLC-MS chromatogram of *V. gracilis* leaf decoction. Numbers above the peaks represent the predominant compounds (in the circle) and their respective retention time (below the circle). (1) valproic acid, (2) 3-hydroxy-3 methyl butanoate; (3) (3b, 16a, 20R)-25-Acetoxy-3, 16, 20, 22-tetrahydroxy-5-cucurbiten-11-one 3-glucoside; (4) Dipropylene glycol methyl ether acetate; (5) 1-Naphthylbis (9-anthryl) methylcation; (6) Carthamoleusterone; (7) 3-[(2-carboxyacetyl) oxymethoxy]-3-oxopropanoic acid 3-[[3-[5-[3-[(2-carboxyacetyl) oxymethoxy]-3-oxopropanoyl] oxy-2, 4-dihydroxypentoxy]-3-oxopropanoyl] oxymethoxy]-3-oxopropanoic acid; 2-(oxiran-2-ylmethyl) oxirane.

with higher doses of decoction (50 and 100 g/L).

Phytochemical constituents of V. gracilis decoction

The phytochemical analysis using UPLC-MS revealed 26 compounds in *V. gracilis* leaf decoction with molecular mass ranging from 60-1027. The chromatogram is depicted in Figure 7 and the compound identities are shown in Table 1. Among all identified substances, 7 compounds were exhibiting

Table 1. Phytochemical compounds in *V. gracilis* leaf decoction revealed by UPLC-MS

No	RT Time	Measured mass	Mol Formula $[M-H]^+$	Compound name
1	1.26	222.0186	$C1_0H_6O_6$	2, 4, 5-trihydroxy-7, 8-dioxonaphthalen-1-olate
2	1.37	144.1019	$C_8H1_6O_2$	valproic acid
3	1.44	215.0661	$C_{13}H_{11}O_3$	phenyl 2-hydroxybenzoate, Phenyl salicylate
4	2.29	167.0712	$C_5H_{11}O_6$	Ribonic acid, 2, 3, 4, 5-tetrahydroxy pentanoic acid
5	2.57	167.0693	$C_9H_{11}O_3$	Ethyl paraben
6	3.47	795.2471	$C_{40}H_{43}O_{17}$	(2S, 3S)-2, 3-Dihydro-2-[2-hydroxy-4-(beta-D-glucopyranosyloxy) phenyl]-3-(3, 5-dihydroxyphenyl) -4-[2-[4-(beta-D-glucopyranosyloxy) phenyl]ethenyl]benzofuran-6-ol
7	4.04	118.0532	$C_5H_{10}O_3$	3-hydroxy-3 methyl butanoate
8	4.57	181.0866	$C_{10}H_{13}O_3$	Propyl 4-hydroxybenzoate
9	5.06	333.1321	$C_{21}H_{17}O_4$	3', 4'-dimethoxy-alpha-naphthoflavone
10	5.45	711.4428	$C_{38}H_{63}O_{12}$	(3b, 16a, 20R)-25-Acetoxy-3, 16, 20, 22-tetrahydroxy-5-cucurbit-en-11-one 3-glucoside
11	5.89	60.0039	$C_2H_4O_2$	Acetic acid
12	5.98	371.3079	$C_{25}H_{39}O_2$	6, 6, 9-trimethyl-3-(3-methyloctan-2-yl)-7, 8, 9, 10-tetrahydrobenzo[c]chromen-1-ol
13	7.45	191.1267	$C_9H_{19}O_4$	Dipropyleneglycol methyl ether acetate
14	7.54	494.1963	$C_{39}H_{26}$	1-Naphthylbis (9-anthryl) methylcation
15	7.61	527.1743	$C_{38}H_{23}O_3$	4-phenylethynylnaphthalic anhydride
16	8.51	457.187	$C_{21}H_{29}O_{11}$	[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-[[(2R,3R,4R,5R,6S)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl] (E)-3-phenylprop-2-enoate
17	9.25	273.1143	$C_{12}H_{17}O_7$	Arbutin
18	9.72	311.1432	$C_{19}H_{19}O_4$	5-[6-hydroxy-5-(3-methylbut-2-enyl)-1-benzofuran-2-yl]benzene-1,3-diol
19	9.8	1027.7008	$C_{56}H_{99}O_{16}$	ethane;2-O-[3-(2-methoxycarbonyl cyclohexanecarbonyl)oxy-2-methyl propyl] 1-O-methyl cyclohexane-1, 2-dicarboxylate; 7-oxabicyclo[4. 1. 0]heptanylmethyl 4-hydroxy-3-methylcyclohexane-1-carboxylate; 1-[[4-(oxiran-2-ylmethoxymethyl) cyclohexyl] methoxy] butanol
20	10.51	337.3269	$C_{22}H_{41}O_2$	(13Z, 16Z)-docosa-13, 16-dienoic acid
21	11.15	550.3538	$C_{28}H_{45}O_8$	Carthamoleusterone
22	13.41	853.5585	$C_{61}H_{73}O_3$	5-propan-2-yl-1, 3-benzodioxole; 1-propanyl naphthalene; 2-propanyl naphthalene; 1-propan-2-yl naphthalen-2-ol; 1,3,5-trimethyl-2-propan-2-ylbenzene
23	15.21	645.6215	$C_{39}H_{81}O_6$	Lauric acid
24	16.13	411.3606	$C_{29}H_{47}O$	4, 4-Dimethyl-5 alpha-cholesta-8, 14, 24-trien-3 beta-ol
25	16.99	637.3809	$C_{42}H_{53}O_5$	Norethindrone acetate and ethinyl estradiol
26	17.36	861.1624	$C_{31}H_{41}O_{28}$	3-[(2-carboxyacetyl) oxymethoxy]-3-oxopropanoic acid; 3-[[3-[5-[3-[(2-carboxyacetyl) oxymethoxy]-3-oxopropanoyl]oxy-2, 4-dihydroxypentoxy]-3-oxopropanoyl] oxymethoxy]-3-oxopropanoic acid; 2-(oxiran-2-ylmethyl) oxirane

substantial peaks (indicating their concentration), namely 3-hydroxy-3methylbutanoate (RT 4.04), (3b,16a,20R)-25-Acetoxy-3,16,20,22-tetrahydroxy-5-cucurbiten-11-one 3-glucoside (RT 5.45), 1-Naphthylbis(9-anthryl)methylcation (RT 7.54), Dipropyleneglycol methyl ether acetate (7.45), 3-[(2-carboxyacetyl) oxymethoxy]-3-oxopropanoic acid;3-[[3-[5-[3-[(2-carboxyacetyl)oxymethoxy]-3-oxopropanoyl] oxy-2,4-dihydroxypentoxy]-3-oxopropanoyl]oxymethoxy]-3-oxopropanoic acid;2-(oxiran-2-ylmethyl) oxirane (RT 17.36), Carthamoleusterone (RT 11.15), and valproic acid (RT 1.37).

Discussion

Our present study revealed a protective effect of *V. gracilis* leaf decoction on sexual function and testis in alloxan-induced diabetes mice. Despite failing to improve blood glucose profile and body weight, *V. gracilis* leaf decoction sustained intense sexual activities while attenuating mass reduction, MDA accumulation and histopathological alterations in the testicle tissues. In addition, the decoction sustained the Hb level, RBC count, and HTC value.

Individuals with sexual dysfunction experience various symptoms including lower sexual desire, decreased physical pleasure, and lack of arousal and orgasm thereby precluding sexual satisfaction [18]. On the other hand, intense sexual activities reflect normal sexual function. A study in streptozotocin-induced diabetic rats [19] found that diabetic progression caused a profound decline in the sexual activities of male rats, including longer latency of mounting, intromission, and interval of post-ejaculatory. Moreover, the frequencies of mounting and intromission were profoundly reduced and the ejaculatory latency was shortened. The findings are in line with our present results showing that diabetic mice exhibited a lower incidence of sexual activities toward estrous females. In contrast, diabetic individuals treated with *V. gracilis* leaf decoction sustained high sexual activities to be comparable with those in the non-diabetic group.

The beneficial effect exerted by *V. gracilis* decoction against the reduction of sexual activities in diabetic mice could be attributed to the protection of the testis. A study in type 1 diabetic rats demonstrated that a chronic hyperglycemic state promoted severe impairments in the testicular tissues and declined testosterone levels [20]. Likewise, our present study in mice also found that diabetes induced apparent histopathological alterations in the testis including the destruction of tubulus seminiferous and increment of necrotic cells both in the tubulus and in interstitial tissues. However, mice treated with lower doses, but not a higher dose, of *V. gracilis* leaf decoction had higher testicle organ mass than those without any treatments. In addition, the *V. gracilis* decoction attenuated diabetic-induced histopathological alterations in the tubulus seminiferous and reduced the number of necrotic cells in the testis in a dose-dependent manner. However, a higher dose of decoction worsened the pathological alterations in the testis of diabetic mice, suggesting a biphasic effect of *V. gracilis*. Although it remains to be elucidated in the future studies, we speculate that at a certain concentration, some phytochemical compounds in the decoction could cause toxicity on the testis. One of the compounds detected in the *V. gracilis* leaf decoction namely valproic acid is suggested to be involved in this case. A study in rats [21] revealed that ten-day administrations of valproic acid caused dramatic alterations in the testicular and epididymal histology. It was also found that valproic acid promoted the disintegration of germ cells and raised the intercellular spaces of epithelial tissues in tubulus seminiferous. Hence, at lower doses of given decoction, the toxic effect of valproic acid might be masked by the other beneficial compounds in *V. gracilis*, thereby attenuating the testicular damages caused by diabetic progression.

Chronic hyperglycemia in diabetes has been suggested to increase lipid peroxidation and subsequent outcomes including elevation of free radicals in various tissues [22]. A study reported that oxidative stress was elevated in the testicle tissue of streptozotocin-induced diabetic rats leading to marked degeneration of tubulus seminiferous, apoptosis of Leydig cells in the testis, and inflammation of epithelial tissue in the epididymis [23]. In our study, it was found that *V. gracilis* decoction reduced oxidative stress in the testis, suggesting an antioxidative effect. Although the antioxidant activity was not determined directly, some compounds detected in the decoction of *V. gracilis* particularly arbutin and lauric acid are suggested to act as antioxidants. *In vitro* assays indicated a strong antioxidant activity of arbutin [24]. Moreover, in another experiment using diabetic rats [25], it was demonstrated that lauric acid has a modulatory role on oxidative stress and improves endogenous antioxidant enzymes.

Chronic exposure of blood cells to hyperglycemia has been documented to cause a reduction in RBC count, Hb and HTC levels [26,27]. This condition could also contribute to the progression of sexual dysfunction and testicle damage due to a limited supply of oxygen. Similarly, our study also found that alloxan-induced diabetic mice had lower RBC count, Hb and HTC values. In contrast, the administration of *V. gracilis* decoction particularly at a lower dose could sustain hematological profiles to be comparable with those in non-diabetic mice. This beneficial effect could be mediated by the antioxidant activities of compounds in the *V. gracilis* leaf decoction thereby precluding substantial disturbance in erythropoiesis. Another study

also showed that abnormal blood values in people with diabetes are associated with pathological alterations in the kidney [28]. Accordingly, an improvement in RBC and Hb values may reflect kidney health, acknowledging the critical role of the kidney in producing erythropoietin to stimulate the synthesis of red blood cells in the bone marrow. Unfortunately, in our study, neither the erythropoiesis nor the kidney structure and functions were investigated. As a result, it remains unknown whether the beneficial effect of a lower dose of *V. gracilis* decoction is mediated through the mechanism involving kidney protection.

Although it exerted a noticeable protective effect on sexual vitality and testicular tissue, *V. gracilis* decoction failed to prevent diabetes progression in mice. We found that the blood glucose levels in decoction-treated mice remained at a hyperglycemic state and their body weight was also less increased. It may suggest that a protective effect of *V. gracilis* on sexual functions is independent of its effect on blood glucose regulation. Specifically, the attenuation of sexual dysfunction and pathological alterations of testis by *V. gracilis* decoction is not mediated by the mechanisms involving pancreatic tissue regeneration or protection. Thus, *V. gracilis* decoction is not effective in alleviating diabetes progression, but specifically useful to protect sexual functions and testis against diabetes. We propose that *V. gracilis* decoction protects the testis by reducing oxidative stress and its subsequent outcomes including inflammation and apoptosis. Further study is warranted to validate this speculation.

We detected various phytochemical compounds in the leaf decoction of *V. gracilis*. Some compounds are common plant secondary metabolites namely valporic acid, ribonic acid, ethylparaben, (3b, 16a, 20R)-25-Acetoxy-3, 16, 20, 22-tetrahydroxy-5-cucurbiten-11-one 3-glucoside, acetic acid, arbutin, carthamoleusterone and lauric acid. Previous general screenings on the leaf extract of *V. gracilis* also detected some groups of metabolites including alkaloids, glycoside, flavonoids, saponins, tannins and terpenoids [12,13]. Unfortunately, the underlying mechanisms of the compounds in exerting their beneficial or toxic effects on sexual and non-sexual vitalities are largely unraveled. Future studies using in silico methods (for instance molecular dockings) may shed further mechanistic aspects of *V. gracilis* as a candidate for potent vitality drugs. In addition, various extraction and fractionation techniques are required to further elucidate the other compounds as potent drug candidates.

We propose several plausible underlying mechanisms of *V. gracilis* leaf decoction in exerting its protective effect on sexual vitality and testis against diabetes. Firstly, bioactive compounds in the *V. gracilis* leaf decoction (particularly arbutin and lauric acid) may act as the exogenous antioxidants to counteract hyperglycemic-induced oxidative stress in the testis. At the same time, the existence of exogenous antioxidants may also enhance the endogenous antioxidants to protect cells against damage. Secondly, *V. gracilis* may protect hematopoietic cells thereby sustaining RBC and Hb production. As a result, oxygen is adequately supplied to the testis ensuring functional cellular activity and regeneration. Thirdly, *V. gracilis* may also inhibit hyperglycemic-induced activation of apoptosis signaling pathway in the testis. Consequently, the cellular damage caused by hyperglycemia in the testis could be minimized. This speculation is in line with a previous report [29] showing that *V. gracilis* extract effectively reduced apoptosis in mice lungs. Another possibility is that the compounds in the *V. gracilis* leaf decoction may promote testosterone production and the expression of its receptor in the testis. As a result, sexual function remained well sustained under diabetic conditions.

In brief, our present study, based on behavioral, biochemical and histopathological observations, provided the convinced scientific evidence for the beneficial effect of *V. gracilis* leaf decoction on protecting sexual vitality against diabetes. Hence, it supports the traditional practice of using *V. gracilis* leaf decoction to enhance vitality. Moreover, this study clearly indicated that the dose of decoction should be considered properly to hinder its detrimental side effects, including testicular degeneration. In addition, this study also reported phytochemical compounds of the *V. gracilis* leaf decoction that may participate in protecting sexual vitality. However, our current study also has some limitations. Firstly, we did not determine the sperm count and sperm viability under the *V. gracilis* leaf decoction treatment. In addition, we did not quantify the apoptotic cells in the testis. Furthermore, we did not provide data of testosterone levels or endogenous antioxidants including catalase, superoxide dismutase and glutathione in the testicular tissue. Our study was carried out in a relatively short term of treatment (28 days), thereby precluding us to clarify whether the protective effect of *V. gracilis* decoction could persist for a longer period. Further investigations employing molecular approaches and using purified compounds are required to define the mechanistic aspects of the *V. gracilis* leaf in supporting sexual vitality against diabetes and associated diseases. Moreover, toxicity tests are absolutely needed to clarify the safety of the *V. gracilis* as a candidate of the drug in the future.

Conclusion

In conclusion, our study revealed that leaf decoction of *V. gracilis*, particularly at the doses of 25 and 50 g/L, could effectively sustain sexual vitality, prevent oxidative stress and histopathological alterations in

the testis while stabilizing blood values, despite failing to reduce blood glucose level in alloxan-induced diabetic mice. However, a higher dose (100 g/L) exhibited pathological effects on testis and blood values. Hence, considering a proper dosage, leaf decoction of *V. gracilis* could be formulated as natural drugs for male sexual vitality and testicle protection against diabetes.

Conflict of Interests
The authors declare no conflict of interest.

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