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Research Article

Effects of *Gynura procumbens* on Sperm Quality and Testosterone Level in Streptozotocin-induced Type 1 Diabetic Rats

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ABSTRACT

Diabetes mellitus have been shown to bring about deteriorating effects on the male reproductive system due to increased oxidative stress. *Gynura procumbens*, an herb with known anti-diabetic properties, was used in this investigation to determine the effect of its aqueous and ethanolic extracts on sperm quality and testosterone level of male streptozotocin-induced Type 1 diabetic rats. Metformin and glibenclamide were used as control drugs in this study. Diabetic rats force-fed with 150 mg/kg of ethanolic *G. procumbens* extract showed dramatic increase in sperm count (206.89%) and motility as well as testosterone level (16.71%), along with decrease in fasting blood glucose level (38.71%) and sperm mortality (57.62%) when compared to the controls. Treatment with aqueous *G. procumbens* extract showed similar results with 23.38% and 31.14% decrease in blood glucose level and sperm mortality respectively, coupled with improvement in testosterone level (8.60%), sperm count (124.79%) and motility. The present data indicate that traditional use of *G. procumbens* as an anti-hyperglycemic agent is justified and may be beneficial for male diabetic patients that suffer from sexual dysfunction as a side effect of prolonged hyperglycemia seen in Type 1 diabetic patients.

Key words: *Gynura procumbens*, diabetes mellitus Type 1, sperm quality, testosterone

INTRODUCTION

Diabetes mellitus brings about deleterious effect on male reproductive system due to disturbances in spermatogenesis as well as erectile and ejaculation dysfunction[1]. It has been estimated that 90% of male diabetic patients suffer from sexual dysfunction, which includes infertility due to testicular dysfunction associated with prolonged hyperglycemia[2]. Oxidative stress (OS) has been implicated as a key contributor in the pathogenesis of multiple diabetic complications, mediated by increased amount of oxygen-derived oxidants known as reactive oxygen species (ROS)[3]. Numerous experimental evidences also have elucidated a potential relationship between oxidative damage in testis or sperm and testicular dysfunction leading to male infertility[4].

Hyperglycemia leads to the increased production of ROS via at least four different routes: increased glycolysis; intercellular activation of the sorbitol pathway; auto-oxidation of glucose and non-enzymatic protein glycation[5] which result in an increase in oxidative stress. ROS is known to be involved in the normal physiological function of human sperm but its excess generation in diabetic condition could lead to the impairment of testicular antioxidant defense mechanism[6]. Sperm are particularly susceptible to oxidative damage due to their polyunsaturated fatty acid (PUFA)-rich plasma membranes[7], which leads to the decrease in sperm membrane fluidity and consequently, sperm motility observed in diabetic subjects[8]. In addition, oxidative stress-mediated DNA and chromatin breakages are also involved in the pathogenesis of infertility in diabetic patients. Direct effect of ROS on the mitochondrial normal functions results in decreased energy supply to the sperm[9]. In prolonged diabetes, the presence of excess blood glucose and its metabolites and ineffective gonadotropin stimulation or resistance could lead to the lowering of plasma testosterone level[10]. Testicular cells exhibit marked decreases in the number and function of Leydig cells, marked by alterations in biochemical marker expression such as GLUT-3 hexose transporter, insulin-like growth factor 1 (IGF-1), androgen receptors and tyrosine phosphorylation, all of which are correlated with the decrease in serum testosterone, insulin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels, leading to impaired spermatogenesis[11].

Gynura procumbens (Lour.) Merr (family Compositae), locally known as "Sambung nyawa" or purple passion vine, is native to the Southeast Asia region, particularly Malaysia, Indonesia and Thailand. This herb has been used traditionally to treat various ailments such as rashes, inflammation, fever, hypertension, migraines, kidney problems, constipation, cancer and diabetes mellitus[12]. Researches shown that *G. procumbens* possesses anti-inflammatory[13], anti-hypertensive[14,15], anti-hyperlipidemic[16], anti-oxidative[17,18] and profertility[19,20] properties. A study by Zurina et al.[21] demonstrated that hypoglycemic action brought about by the treatment of *G. procumbens* leaves extract in RIN-5F cell line involves

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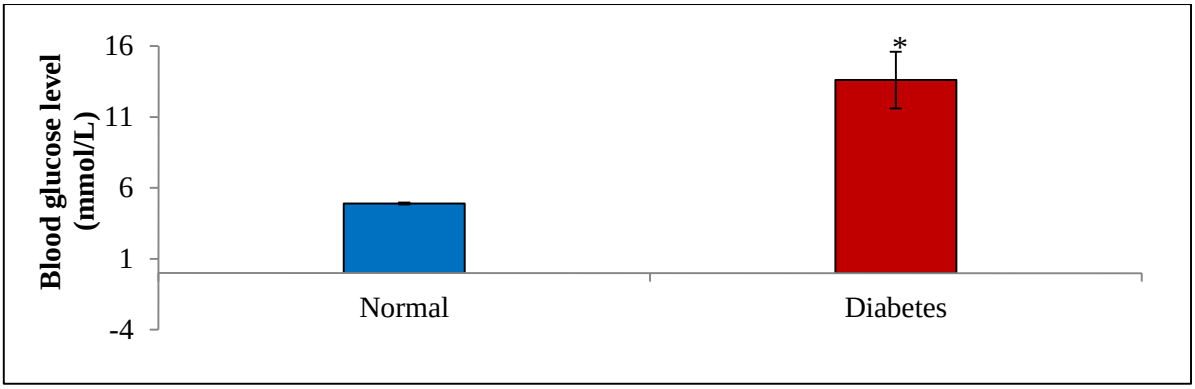

Figure 1: Effect of STZ induction on blood glucose level in Type 1 diabetic rats (n = 5), (P < .05).

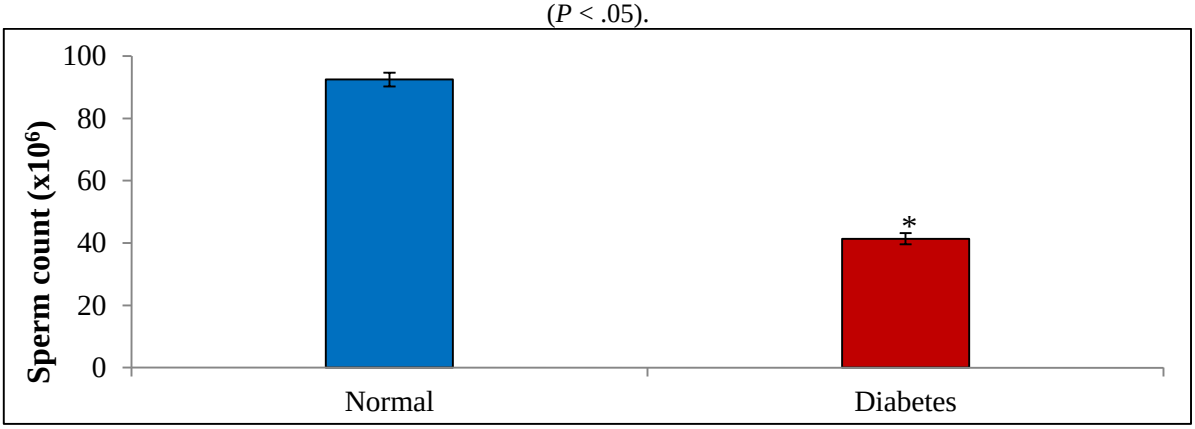

Figure 2: Effect of *G. procumbens* extracts on blood glucose level in Type 1 diabetic rats (n = 5), (P < .05).

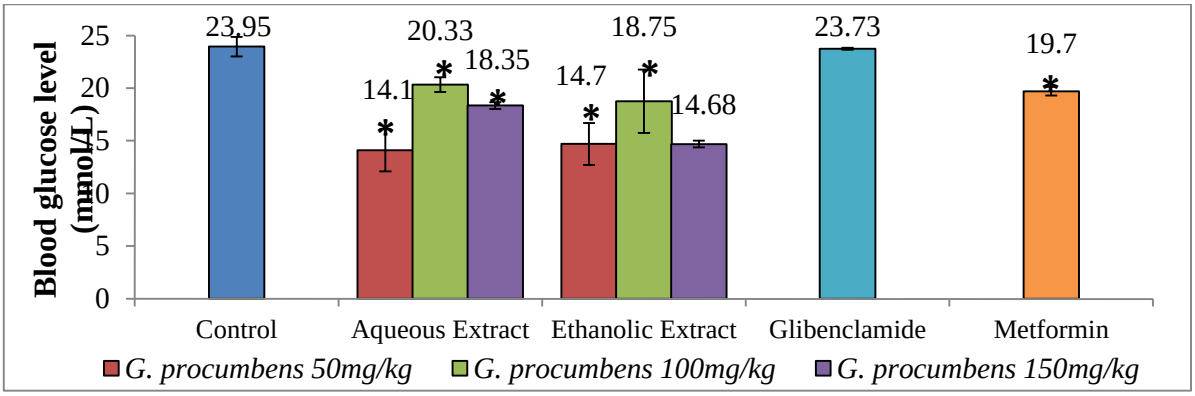

Figure 3: Effect of STZ induction on sperm count in Type 1 diabetic rats (n = 5), (P <0.05).

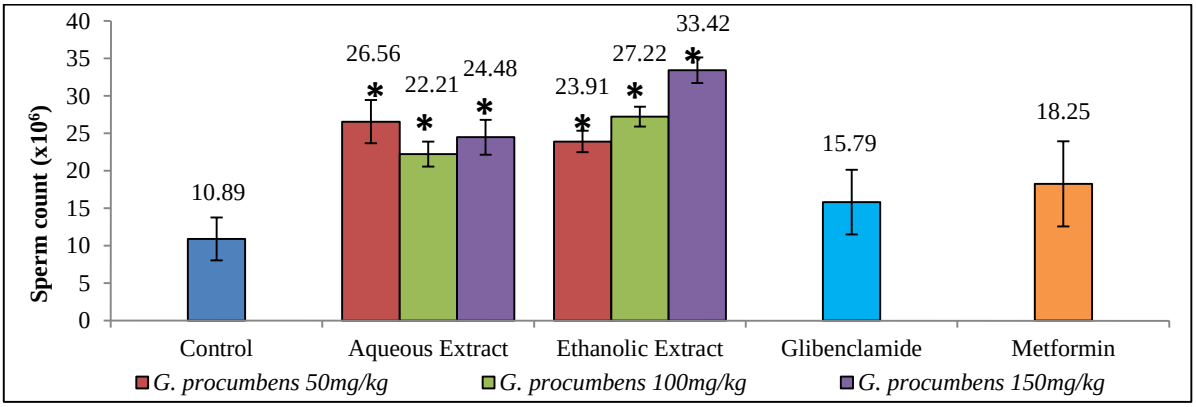

Figure 4: Effect of *G. procumbens* extracts on sperm count in Type 1 diabetic rats (n = 5), (P < 0.05).

extrapancreatic mechanisms due to absence of effects on the glucose-stimulated insulin secretion.

Among the identified components present in n-butanol fraction of *G. procumbens* methanolic leaves extract that could possibly confer its anti-hyperglycemic ability includes flavonoids, saponins, terpenoids and tannins[22]. On the other hand, the effectiveness of *G. procumbens* extract in playing its role as radical scavenger and antioxidant could be atrributed to its sterol constituents such as 3-*O*-β-D-glucopyranosyl stigmasterol, 3-*O*-β-D-glucopyranosyl β-sitosterol and 3-*O*-β-D-tetra-*O*-acetylglucopyranosyl β-sitosterol[23]. Many researches have demonstrated the effectiveness of *G. procumbens* as a natural hypoglycemic agent but little is known of its profertility potential on diabetic subjects. This study aims to evaluate the profertility properties of aqueous and ethanolic *G. procumbens* leaves extracts by examining its effects on sperm quality and plasma testosterone levels in male diabetic rats that serve as a model of Type 1 diabetes.

MATERIALS AND METHODS

Animals

A total of 55 male Sprague dawley rats, aged 10 weeks old used in this experiment were provided by Animal House, Faculty of Science & Technology, Universiti Kebangsaan Malaysia. The animals were housed in polypropylene cages, fed a standard rat chow diet and water *ad libitum*, exposed to 12-hour light/dark cycle at room temperature. The animal care and protocol in this investigation conformed to the guidelines set by Animal Ethics Committee of Universiti Kebangsaan Malaysia (UKMAEC) (UKMAEC Approval Number FST/SBB/HALIMAH/24-AUGUST/322-SEPTEMBER-2010-NOVEMBER-2011).

Experimental Design

Rats were randomly divided into nine experimental subgroups: control; treatment with *G. procumbens* ethanolic extract 50, 150 or 150 mg/kg body weight (b.w.); treatment with *G. procumbens* aqueous extract 50, 100 or 150 mg/kg b.w.; glibenclamide 5 mg/kg b.w.; metformin 500 mg/kg b.w. Rats were allowed to be acclimatised to the laboratory environment for two weeks prior to the study. Diabetes was induced via intravenous injection of 55 mg/kg b.w. streptozotocin (dissolved in citrate buffer pH 4.5 before use; Merck KgaA, Darmstadt, Germany) to overnight-fasted rats. Diabetes was confirmed 7 days after the administration using AccuChek® Performa glucometer (Roche Diagnostics GmbH, Germany). Rats with fasting blood glucose level of 14 mmol/L showed signs of established diabetes[25] and were chosen to be given treatments for six weeks. Treatments of *Gynura procumbens* extracts, glibenclamide and metformin were administered via the oral gavage method. For each of the treatment group, the appropriate weight of each substance was measured depending on the dose administered per rat and dissolved in 1 ml of distilled water. Control group rats were given distilled water via similar method during the treatment duration. Rats were sacrificed after treatment period ended.

Gynura procumbens extracts preparation

G. procumbens aqueous extract was prepared through the method of Peungvicha[24]. The leaves sample used in this experiment were obtained from Plant House, School of Biosciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia. Briefly, dried leaves were pounded, mixed with distilled water (1:20) and heated in water bath at 50°C for three hours. The ethanolic extract was prepared by soaking 1 kg of blended fresh leaves in 95% ethanol for seven days with constant stirring[16]. The infusions were filtered, centrifuged and the supernatants obtained were later freeze-dried. The resulting extracts were suspended in distilled water before administration. In this research, preliminary findings have shown that different batches of extracts prepared did not lead to any significant variations on the data as long as the extract preparation methods were followed as strictly as possible.

Sample Preparation

Blood samples used for blood glucose level determination were obtained from tail vein of rats fasted overnight (16 hours). After the rats were sacrificed using chloroform inhalation, blood samples for testosterone analysis were immediately collected via cardiac puncture and deposited into vacutainer tubes containing EDTA and centrifuged at 3000g for 10 minutes. Plasma samples were obtained and kept in -20°C until analysed. Sperm from the cauda epididymis were released by cutting into B.W.W. (Biggers-Whitten-Whittingham) medium. After 15 minutes incubation at 37°C with 5% CO_2, the cauda epididymal sperm sample were subjected to sperm quality assessment, namely for sperm count, motility and mortality[26].

Blood Glucose Level Determination

Blood glucose level was measured at the end of the treatment period prior to sacrifice using AccuChek® Performa glucometer.

Sperm Quality Analysis

Sperm quality parameters were determined based on standard hemocytometric procedures[26] using improved Neubauer haemocytometer (Hausser Scientific Company, Pennsylvania) under light microscope (Leica Microsystems CMS GmbH, Germany). Sperm motility was analysed and reported as mean percentage of motile sperm based on the respective motility grades according to WHO standards[26].

Serum Testosterone Level Analysis

Plasma testosterone level was determined using ELISA by Testosterone EIA kit (Cayman Chemical, Michigan, USA) according to the manufacturer's details[27,28].

Statistical analysis

Data were expressed as mean ± SEM and a paired t test was used to analyse the significance of mean difference between treatment groups and control group using computerised MINITAB 15 software. A value of $P<0.05$ was considered to be statistically significant (marked with the symbol * in Results).

RESULTS

Blood Glucose Level

Table 1: Effects of STZ induction on sperm motility of Type 1 diabetic rats based on sperm motility grades (WHO 1999).

Group	Percentage of sperm motility grade (%)			
	a	b	c	d
Normal	33.78±3.5	18.70±1.0	22.69±2.7	24.83±0.9
Diabetis	18.68±0.69*	16.22±0.91	15.15±1.2	49.95±1.4*

Note: Results are expressed as mean±SEM (n = 5).
* P<0.05 when compared to control group (Students t-test).
a The most progressive sperm movement (≥ 25 µm/s)
b Intermediate movement of sperm (5 – 24 µm/s)
c Slow movement of sperm (< 5 µm/s)
d Immotile sperm

Figure 1 established the hyperglycemia-inducing effect of STZ induction in rats. Diabetic rats fed with *G. procumbens* extracts showed significant (P<0.05) reduction in blood glucose level (Figure 2). Diabetic rats administered with ethanolic extract of *G. procumbens* leaves at 50, 100 and 150 mg/kg b.w. showed 38.62%, 21.71% and 38.71% decrease in blood glucose level, respectively. Treatment of *G. procumbens* aqueous extract at 50, 100 and 150 mg/kg b.w. resulted in 41.13%, 15.11% and 23.38% reduction of blood glucose level in diabetic rats, respectively. Significant (P<0.05) decrease in blood glucose was also observed in diabetic rats treated with metformin (500 mg/kg b.w.) but conversely, no significant changes were seen after the administration of glibenclamide (5 mg/kg b.w.)

Sperm Count

As shown in Figure 3, the STZ induction resulted in decreased sperm count in Type 1 diabetic rats significantly when compared to normal rats. Generally, administration of both aqueous and ethanolic extracts of *G. procumbens* leaves significantly (P<0.05) increased the sperm count of diabetic rats (Figure 4). The highest increment of sperm count can be seen in diabetic rats treated with ethanolic *G. procumbens* leaves extract at 150 mg/kg b.w. where a three-fold increment was observed when compared to control rats. Oral administration of glibenclamide and metformin resulted in noticeable, albeit statistically insignificant, 45% and 67.58% increment in sperm count of diabetic rats, respectively in comparison to the control group.

Sperm Motility

STZ induction significantly decreases sperm motility, as shown in Table 1. The administration of ethanolic *G. procumbens* extracts at all doses and glibenclamide (Table 2) managed to cause significant (P<0.05) increment in the percentage of progressively motile sperm (grade a) and significant (P<0.05) decrease in the percentage of immotile sperm (grade d). On the other hand, treatment of aqueous *G. procumbens* extract at 50 mg/kg b.w. did not produce significant changes in the percentage of sperm motility in any of the four sperm motility grades.

Sperm mortality

Sperm mortality is significantly increased in Type 1 diabetic rats as shown in Figure 5. Based on the data summarised in Figure 6, it is evident that both aqueous and ethanolic *G. procumbens* leaves extracts decrease the percentage of sperm mortality in dose-dependent manners. The highest reduction in sperm mortality can be seen in diabetic rats treated with aqueous *G. procumbens* extract at 50 mg/kg b.w. (58.26%) followed by rats given ethanolic *G. procumbens* extract at 150 mg/kg b.w. (57.62%) when compared to the sperm mortality rate in control group.

Plasma Testosterone Level

Figure 7 indicates the lowering effect STZ induction has on plasma testosterone level. As seen in Figure 8, oral administration of aqueous *G. procumbens* leaves extract (150 mg/kg b.w.) and ethanolic *G. procumbens* leaves extract (50, 100 and 150 mg/kg b.w.) produced significant (P<0.05) improvement on plasma testosterone level of diabetic rats when compared to diabetic control group. No significant increase was observed in plasma testosterone level of metformin- and glibenclamide-treated diabetic rats

DISCUSSION

Based on the data obtained, it was shown that aqueous and ethanolic *G. procumbens* extracts possess the ability to regulate blood glucose level in Type 1 diabetic rats, which concurs with other reports[16,21,29-32]. A parallel study demonstrated that administration of *G. procumbens* leaves aqueous and ethanolic extracts significantly lowered blood glucose and HbA1c levels as well as increased liver glycogen content and activities of enzymes involved in carbohydrate metabolism without affecting plasma insulin levels in diabetic rats[33]. It has been suggested that instead of acting as an insulinotropic agent, *G. procumbens* leaves extracts elicit their effects through extrapancreatic pathways, where these extracts might contain chemical compounds such as kaempferol-3-*O*-glycoside and other glycoside flavonoids[34] that mimic the action of insulin or act by enhancing the effects of insulin at cellular level[29,31]. The extrapancreatic pathways that might be stimulated by these compounds include enhanced transport and utilisation of blood glucose by peripheral tissues, increased activity of glycolytic enzymes in peripheral tissues[32,33] as well as decreased secretion of growth hormone, glucagon and cortisol[34].

The antihyperglycemic effect of *G. procumbens* extracts is at par with metformin, highlighting the potential of *G. procumbens* leaves possess as natural antidiabetic agent. Metformin exerts its effects in lowering the blood glucose by inhibiting carbohydrate metabolism enzymes such as PEPCK and glucose-6-phosphatase that are involved in hepatic gluconeogenesis, aside from increasing peripheral glucose uptake[35]. Flavonoids that are present in plant and herbal extracts have been reported to possess insulin-like

Table 2: Effects of *G. procumbens* extracts treatments on sperm motility of Type 1 diabetic rats based on sperm motility grades (WHO 1999)

Percentage of sperm motility grade (%)	Control	Aqueous extract *G. procumbens*			Ethanolic extract *G. procumbens*			Glibenclamide (5 mg/kg)	Metformin (500 mg/kg)
		50 mg/kg	100 mg/kg	150 mg/kg	50 mg/kg	100 mg/kg	150 mg/kg		
a	11.22±0.92	12.57±0.19	12.35±0.67	11.92±0.84	17.65±1.70*	20.98±0.92*	25.99±1.10*	20.26±1.40*	19.22±2.80
b	14.58±0.60	14.70±0.77	11.41±0.95*	19.49±0.55*	18.22±1.10*	22.67±0.83*	23.01±1.10*	8.07±0.78*	8.16±2.20*
c	22.19±0.94	19.52±3.30	23.29±1.30	20.87±0.64	25.16±1.50	28.97±1.40*	23.08±1.00	29.49±1.50*	20.54±1.60
d	52.01±1.80	53.21±1.80	52.95±2.90	47.72±1.30*	38.97±1.30*	27.38±2.70*	27.92±1.80*	42.18±2.30*	52.08±3.70

Note: Results are expressed as mean±SEM (n = 5).
* P<0.05 when compared to control group (Students t-test).
a The most progressive sperm movement (≥ 25 µm/s)
b Intermediate movement of sperm (5 – 24 µm/s)
c Slow movement of sperm (< 5 µm/s)
d Immotile sperm

actions such as reducing the expression of hepatic glucose-6-phosphatase enzyme via increased tyrosine phosphorylation by insulin receptors and insulin receptor substrate-1 (IRS-1), MAPK protein kinase (MEK) and phosphatidylinositol 3-kinase (PI3K) as well as PI3K-mediated inhibition of PEPCK enzyme[36,37]. In addition, kaempferol, a type of flavonoid glycoside, has the ability to stimulate glycogen synthesis in muscle tissue via PI3K-GSK-3 and MEK-PP-1 pathways[38]. The overall action of flavonoids on carbohydrate metabolisms could provide the answer to the question of how aqueous and ethanolic *G. procumbens* extracts play their part as antihyperglycemic agents.

Oxidative stress has been shown to be dramatically increased in diabetic state due to excessive generation of reactive oxygen species (ROS) and failing oxidative defences[39,40]. About 30-80% of infertility cases stemmed from sperm damage are due to ROS actions[41-43]. Clinical and diabetic animal model researches demonstrated the presence of spermatogenesis dysfunction, atrophy of seminiferous ducts, decreased number and motility of sperm as well as elevated sperm mortality in diabetic subjects[1,44-48].

The presence of small amount of free radicals is in fact essential in the capacitation process during fertilisation. Hydrogen peroxide induces the acrosome reaction to occur which aids in sperm penetration through the zona pellucida layer of the oocytes[9]. In addition, low levels of hydrogen peroxide stimulate the sperm and oocyte fusion by driving tyrosine phosphorylation to take place, ultimately allowing the binding of sperm membrane to zona pellucida surface protein ZP-3. Conversely, excessive generation of ROS leads to the peroxidation of acrosome membrane of sperm, thus disturbing the acrosome reaction and fertilisation process[49,50].

Early research reported that sperm membrane peroxidation by ROS leads to decreased flexibility of the sperm's tail[51], affecting its motility. Sperm membrane is highly susceptible to ROS-induced damages due to its high composition of polyunsaturated fatty acid (PUFA) content, where sperm mitochondrial damage could lead to lack of energy availability and hinders sperm motiliy and movements[52]. Excessive free radicals could also directly cause DNA degradation and induce apoptosis via caspase reaction[53-55] where research has shown that diabetic patients have higher degree of sperm DNA fragmentation in comparison with normal individuals[56].

Administrations of aqueous and ethanolic *G. procumbens* extracts successfully ameliorate sperm quality of Type 1 diabetic rats that deteriorated due to increased testicular oxidative stress that arise as one of systemic complications of diabetic state. Current work mirrors the result obtained in a previous study[20], though the mechanisms that are involved in resulting improved sperm quality after the administration of these extracts remain unknown. Other reports indicated that the presence of certain flavonoids in herbal plants such as kaempferol-3-*O*-rutinoside and astragalin contribute to increased protection against oxidative stress, mediated by free radical scavenging activities, metal ion chelation and deactivation of oxygen singlet[57,58]. On the other hand, another herb, *Zingiber officinale*, also possesses antioxidative and profertility properties that are believed to be mediated by its antioxidant and androgenic active constituents such as zingeron, gingerdiol, zingibren, gingerol and shagaol[59]. These natural antioxidants could potentially protect sperm DNA from fragmentation and lipid membrane contents

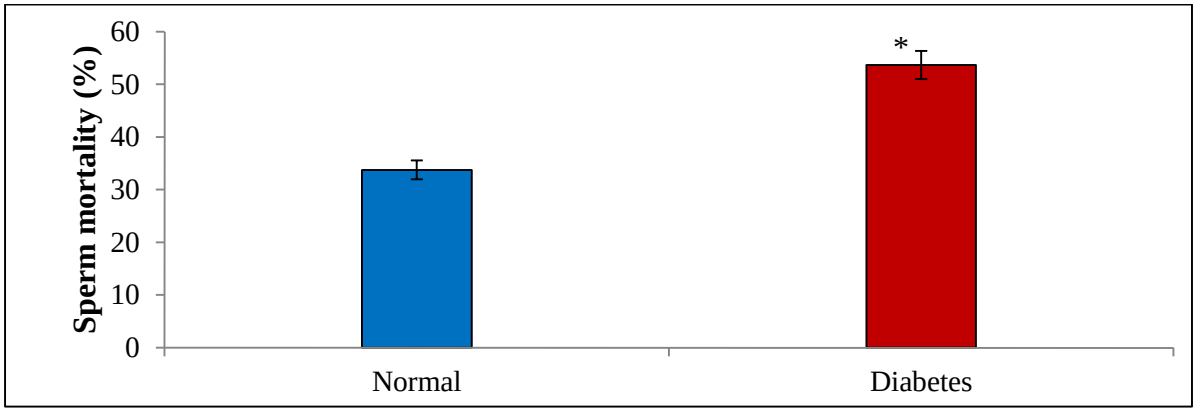

Figure 5: Effect of STZ induction on sperm mortality in Type 1 diabetic rats (n = 5), (P < 0.05)

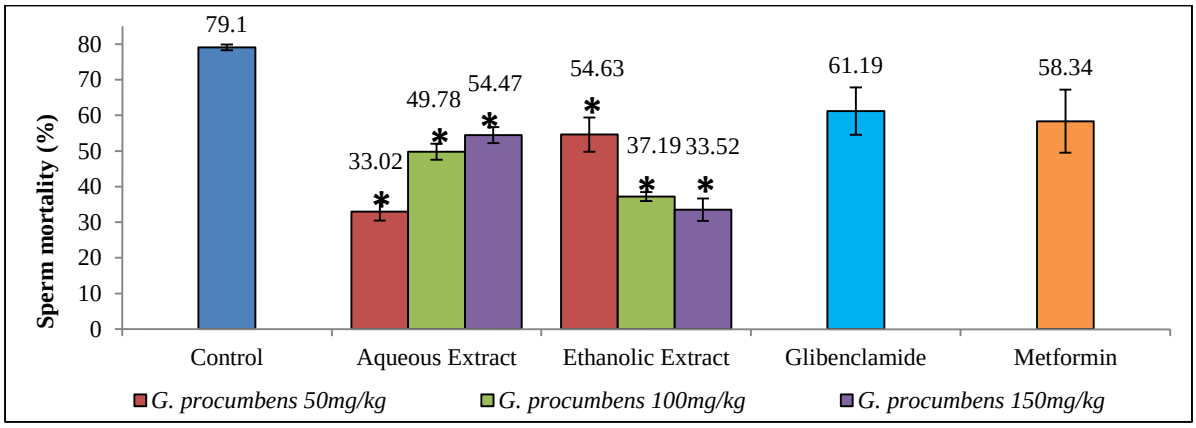

Figure 6: Effect of *G. procumbens* extracts on sperm mortality in Type 1 diabetic rats (n = 5), (P < 0.05).

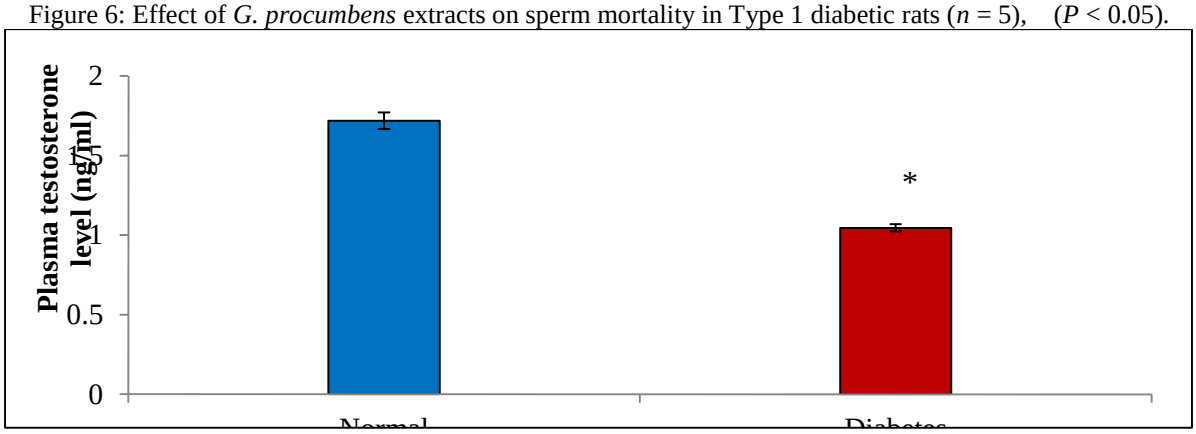

Figure 7: Effect of STZ induction on plasma testosterone level in Type 1 diabetic rats (n = 5), (P < 0.05)

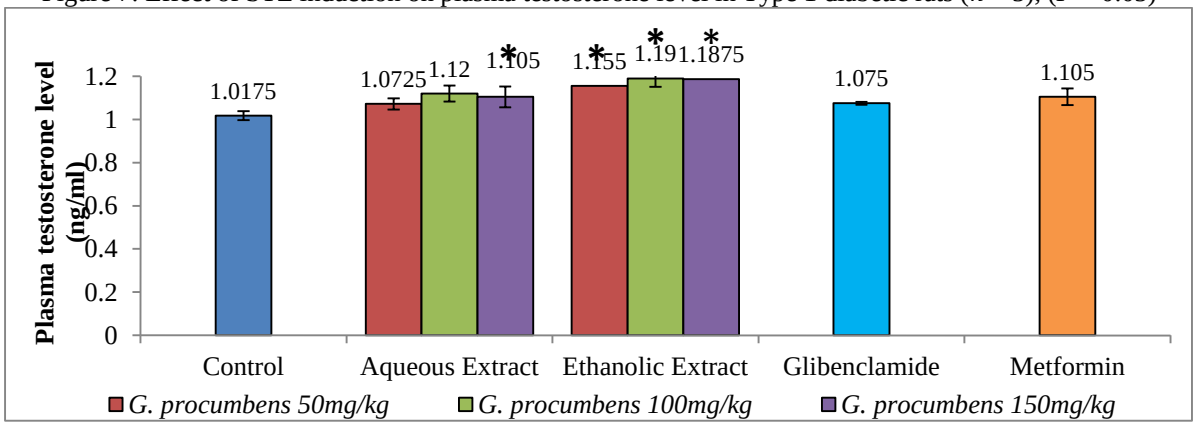

Figure 8: Effect of *G. procumbens* extracts on plasma testosterone level in Type 1 diabetic rats (n = 5), (P < 0.05).

from oxidative damages, thus improving male fertility by increasing sperm overall quality[60].

The present study shows increased plasma testosterone level in insulin-dependent diabetic rats when fed with aqueous and ethanolic *G. procumbens* extracts. Unfortunately, there are no current data that could elucidate the mechanism of profertility action of this herbal plant. The increment in testosterone level after administration of *G. procumbens* extracts observed in this study could be due to the elevated activities of main androgenic enzymes such as Δ5,3β-hydroxysteroid dehydrogenase (Δ5,3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD), where both are involved in the metabolism of testosterone synthesis[61]. Studies on other plants, for example *Ajuga iva*, demonstrated that the synergy action of calcitriol and phytoestrogens are responsible in strengthening the cell's antioxidant system, ultimately maintaining the levels of testosterone and estrogens[62,63]. Intake of plant-derived compounds that are physically similar to steroids such as phytoestrogens and flavonoids are proven to increase the level of aromatase enzyme and estrogen receptor expressions in testes, where both are respectively important in estradiol production and signalling[64]. The delicate balance of testosterone and estradiol levels is imperative in ensuring the perfect workings of spermatogenesis process and maintaining optimum sperm quality[65].

A study by Puangpronpitag et al.[18] has shown that ethanolic extract of *G. procumbens* leaves possesses the highest polyphenol and flavonoid content in comparison to aqueous, hexane, acetonitrile, chloroform and methanolic extracts. Phenolics in ethanolic *G. procumbens* extract are believed to work in a more synergic and effective manner in neutralising free radicals and oxidants compared to other types of extract. In therapeutic point of view, alcoholic-based plant extracts have been known to be more competent antioxidant agents than their aqueous counterparts[66] due to the presence of more dissolved oxindole alkaloids and bioactive components in alcoholic extract preparation. This may explain the better improvement in sperm quality experienced by diabetic rats given the ethanolic *G. procumbens* extract when compared to aqueous extract-fed diabetic Type 1 rats, particularly in the sperm count parameter.

CONCLUSION

As a conclusion, the profertility effect exerted by *G. procumbens* extracts bring about increases in sperm quality and plasma testosterone level in Type 1 diabetic rats, where the flavonoids present in these extracts could act by strengthening the impaired antioxidant defence system typically found in diabetic condition due to prolonged hyperglycemia. Further researches involving isolating and identifying the particular components of the plant that are responsible in conferring its antidiabetic and profertility abilities could be useful in finding a remedy for infertility in male diabetic patients.

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