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Original Article

Effects of *Black ginger (Kaempferia parviflora)* on the testicular function in streptozotocin-induced diabetic male rats

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Abstract The present study aimed to investigate the efficacy of *Black ginger (Kaempferia parviflora; KP)* on testicular function in streptozotocin (STZ)-induced diabetes rats (DM). Six-week-old male Wistar rats were used in this experiment. All rats were randomly divided into six group; group 1: Normal group, group 2: DM (control), group 3: DM rats received glibenclamide 5 mg/kg, group 4-6: DM rats received KP 140, 280 and 420 mg/kg, respectively. DM was induced by intraperitoneal injection of STZ (60 mg/kg). All animals were treated for 6 weeks. Blood glucose, epididymal sperm parameter, testicular microstructure and testosterone level were evaluated. The result showed that KP treatment has no effect on blood glucose in DM rats. KP treatment showed significantly increased sperm density, serum testosterone and ameliorates testicular structure in DM rats ($p < 0.05$). The present study indicates that the aphrodisiac properties of KP could improve of sperm density, testosterone level and testicular function in STZ-induced diabetes rats.

Keywords; Diabetes mellitus, Testes, Testosterone, *Kaempferia parviflora*

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บทความต้นฉบับ

**ผลของกระชายดำ (*Kaempferia parviflora*) ต่อการทำงานของอวัยวะใน
หนูแรทเพศผู้ที่ถูกเหนี่ยวนำให้เป็นโรคเบาหวานด้วย Streptozotocin**

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บทคัดย่อ การศึกษาครั้งนี้มีวัตถุประสงค์เพื่อศึกษาประสิทธิภาพของกระชายดำ (*Kaempferia parviflora*; KP) ต่อการทำงานของอวัยวะในหนูแรทเพศผู้ที่ถูกเหนี่ยวนำให้เป็นโรคเบาหวานด้วย Streptozotocin ทำการศึกษาในหนูแรทเพศผู้ สายพันธุ์ Wistar อายุ 6 สัปดาห์ หนูทุกตัวถูกแบ่งเป็น 6 กลุ่มด้วยวิธีการสุ่มประกอบด้วย กลุ่มที่ 1 หนูปกติ (ควบคุม) กลุ่มที่ 2 หนูเบาหวาน กลุ่มที่ 3 หนูเบาหวานได้รับยา glibenclamide ขนาด 5 มก/กก กลุ่มที่ 4-6 หนูเบาหวานที่ได้รับสารสกัดกระชายดำขนาด 140, 280 และ 420 มก/กก ตามลำดับ หนูกลุ่มเบาหวานถูกเหนี่ยวนำให้เป็นโรคเบาหวานด้วยวิธีการฉีด streptozotocin ขนาด 60 มก/กก เข้าช่องท้อง หนูทุกกลุ่มได้รับการป้อนสารเป็นเวลา 6 สัปดาห์ จากนั้นทำการตรวจระดับน้ำตาลในเลือด คุณภาพของอสุจิบริเวณอภิติเดมิส ลักษณะทางจุลกายวิภาคของอวัยวะและระดับฮอร์โมนเทสโทสเตอโรน ผลการศึกษาพบว่า สารสกัดกระชายดำไม่มีผลต่อระดับน้ำตาลในเลือด นอกจากนี้พบว่าสารสกัดกระชายดำมีผลเพิ่มความหนาแน่นของอสุจิ ระดับฮอร์โมนเทสโทสเตอโรนและลักษณะทางจุลกายวิภาคของอวัยวะในหนูที่ถูกเหนี่ยวนำให้เป็นโรคเบาหวานอย่างมีนัยสำคัญทางสถิติ ($p < 0.05$) จากผลการศึกษาแสดงให้เห็นว่าคุณสมบัติการเพิ่มสมรรถภาพทางเพศของกระชายดำสามารถเพิ่มความหนาแน่นของอสุจิ ระดับฮอร์โมนเทสโทสเตอโรนและการทำงานของอวัยวะในหนูที่ถูกเหนี่ยวนำให้เป็นโรคเบาหวานด้วย streptozotocin

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Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder that continues to be a major of global health problem (Jangir and Jain, 2014; Roehrs et al., 2014; Tsounapi et al., 2012). The prevalence is rapidly increasing and estimate 366 million people living with the disease by 2030 (Mulholland et al., 2011). DM affects many organs such as heart, kidney, eye, peripheral nerves and male reproductive organs (Bayram et al., 2015). The recently report that decrease male reproductive function is common complication in both diabetic men and experimental animal model (Bal et al., 2011). The number of young patients with DM type 1 is rising drastically. Therefore, infertility of young patients has become concern (Tsounapi et al., 2012). Previous report indicated that DM has effects on accessory sex organs, spermatogenesis, steroidogenesis, semen quality, erectile dysfunction and sexual behaviour (Abbasi et al., 2013; Heeba and Hamza, 2015; Nasrolahi et al., 2013). It is also suggested that DM lead to the increase oxidative stress and impairment of antioxidant enzymes, thus resulting in male reproductive dysfunction (Bal et al., 2011; Roehrs et al., 2014; Tsounapi et al., 2012). *Kaempferia parviflora* Wall. Ex. Baker or "Krachai dum", is belong to a family of Zingiberaceae. It has been used a folk medicine in Thailand for treating various illness including allergy, fatigue, general pain, gastrointestinal disorder, allergy, fatigue, general pain, gastrointestinal disorder, promote health, and male sexual

dysfunction (Murata et al., 2013; Trisomboon et al., 2008; Wattanathorn et al., 2012). This plant has been increasingly used as an alternative medicine. Moreover, the toxicological study have shown that administration of KP for 60 days had no effect on hemoglobin, WBC, differential cell count, and hepatic and kidney function (Sudwan et al., 2006). Recent finding showed that KP contains chalcone derivatives, flavonoids and flavonoid glycosides that contain many methoxyl groups. The various of polymethoxyflavone substances including 7-dimethoxyflavone; 5-hydroxy-3; 5-hydroxy-3,7,4'-trimethoxyflavone; 5-hydroxy-7-methoxyflavone; 3,5,7-trimethoxyflavone; 5-hydroxy-7,4'-dimethoxyflavone; 5-hydroxy-3,7,3',4'-tetramethoxyflavone; 5,7,4'-trimethoxyflavone; 3,5,7,4'-tetramethoxyflavone and 5,7,3',4'-tri methoxyflavone; were a major compound in KP roots (Akase et al., 2011; Shimada et al., 2011; Sutthanut et al., 2007; Trisomboon et al., 2008; Youn et al., 2016). The pharmacokinetic studies of KP extract indicated that its low oral bioavailability, methoxyflavones achieved the maximum concentration within 1-2 hr after oral administration and their $T_{1/2}$ is 3-6 hr (Mekjaruskul and Sripaidkulchai, 2015). The aphrodisiac properties of KP were promoted and have long been used among Thai men. However, there were no laboratory results to support it potential in diabetes animal model. The aim of this study was to investigate the effect of KP on testicular function in streptozotocin-induced diabetes rats.



Materials and Methods

Plant material

The KP rhizomes were obtained from Loei province, Thailand, and identified by Assoc. Prof. Monchan Maketon, Department of Zoology, Faculty of Science, Kasetsart University, Thailand. The rhizomes were slice, dried at 60°C for 24 hr and ground. The KP powder was extracted with 50% ethanol in a Soxhlet apparatus and evaporated by rotary evaporation, lyophilized. The lyophilized KP was stored at -20°C until use.

Animals care and experimental design

Thirty-six adult male Wistar rats weighing about 180-200g were obtained from the National Laboratory Animal Center, Mahidol University, Thailand. The animals were housed individually in standard polypropylene cages, which maintained under controlled conditions of light cycle (12 hr:12 hr light:dark), room temperature (25±2°C) and relative humidity (60-70%) with free access water and rat chow. Rats were allowed to acclimatize to the laboratory environment for 7 days before starting the experiment. All procedures were accord with the National Institutes of Health, U.S.A., Guide for the care and Use of Laboratory Animals and were conducted according to ethical guidelines of Kasetsart University Research and Development Institute, Kasetsart University, Thailand (ID:OACKU00258).

Diabetes mellitus was induced by single intraperitoneal injection of streptozotocin (Sigma, St. Louis, MO, USA) that was fresh prepared in

dissolved in ice-cold citrate buffer (0.1 M, pH 4.5) at a dose 60 mg/kg. Diabetes was confirmed by a drop of blood taken from the tail for glucose level estimation via blood glucose meter (Accu-Check Active, Roche Diagnostic, Germany). Only animals exhibiting a fasting glucose level greater than 250 mg/dl three days after STZ injection were included in this study. Animals were randomly divided into six groups comprising six animals in each group.

- Group 1: normal nondiabetic rats received vehicle
- Group 2: nontreated diabetic rats received vehicle
- Group 3: diabetic rats treated with glibenclamide at 5 mg/kg
- Group 4: diabetic rats treated with KP extract at 140 mg/kg
- Group 5: diabetic rats treated with KP extract at 280 mg/kg
- Group 6: diabetic rats treated with KP extract at 420 mg/kg

The extract was suspended in distilled water with Tween 80 to prepare a 1% suspension. The study was continued for 6 weeks using an oral gavage tube.

Blood glucose

Fasting blood glucose was monitored once in a week during experiment period by a drop of blood taken from the tail for glucose level estimation via blood glucose meter (Accu-Check Active, Roche Diagnostic, Germany).



Sperm collection and analysis

At the end of the experiment all animal were sacrificed by overdose of pentobarbitone sodium. The testes, seminal vesicle and epididymis were collected, washed in saline and blotted dry with filter paper. The organs were weighted using an electronic balance. The semen was obtained from the caudal epididymis by mincing the epididymis into small pieces and mixing the epididymal fluid with 1 ml of Hanks'balance salt solution prewarmed at 37°C. Sperm parameters such as sperm count, motility and viability were examined by microscope according to the method by Raji *et al.* (2003). Sperm count was done using Neubauer cell counting chamber under 10X magnification. Percentage of sperm viability, morphologically normal and abnormal was accessed by the one-step eosin-nigrosin staining technique. Nonstained cells were considered as alive and in dead cells, stain had passed through the membrane and colored as orange-red. Sperm morphology was evaluated.

Hormonal analysis

Blood samples were collected from posterior vena cava and centrifuge at 2,200g for 15 min at 4°C. The serum was store at -35°C for analysis. The serum Testosterone level was measured by colorimetric method using Testosterone ELISA Kit ab108666 (Abcam, Cambridge, UK) according to the manufacturer's instructions. The sensitivity of the assay was 0.07

pg/ml. The intra- and interassay coefficients of variation were 5.5 and 10.5%, respectively.

Histological evaluation

After being weighed, the testes were immediately fixed in Bouin's solution for 24 h. Tissues were processed according to the standard histological protocol for paraffin embedding and cut into 5- μ m-thick slices. Sections were stained with hematoxylin-eosin (HE). The sections were viewed under a light microscope and photomicrographs were taken. The microscopic structures of the seminiferous tubules, Sertoli cells, Leydig's cells, adventitial cells and the extent of spermatogenesis were evaluated. At least randomly selected 30 round tubules from each animal were measured of the diameter to determine the mean seminiferous tubule diameter (MSTD). The spermatogenesis was categorized based on the Johnson score (Celik *et al.*, 2013). A grade from 1 to 10 was given to each tubule cross-section according to the following criteria: 10=complete spermatogenesis and perfect tubules; 9=many spermatozoa present and disorganized spermatogenesis; 8=only a few spermatozoa present; 7=no spermatozoa but many spermatids present; 6=only a few spermatids present; 5=no spermatozoa or spermatids but many spermatocytes present; 4=only a few spermatocytes present; 3=only spermatogonia present; 2=no germ cells but only Sertoli cells present; 1=no germ cells and no Sertoli cells present.



Statistical analysis

The results were expressed as the mean±SEM. The statistical analysis was performed by one-way ANOVA followed by the post hoc Tukey test using R Project Statistical Computing package (R Core Team, 2016). P values <0.05 were considered as statistically significant.

Results

Effect of KP on blood glucose level

STZ-induced diabetes significantly increased blood glucose level by 2-3 folds compared to the normal control rats ($p<0.05$). However, KP treatment did not have effects on the blood glucose levels in diabetic rats (Table 1).

Effect of KP on reproductive organ weight and serum testosterone level

The inductions of diabetes cause significant decrease ($p<0.05$) in weight of testes, epididymis and seminal vesicle compared to normal rats (Table 2). However, KP-treated diabetic rats had significantly improved testicular weight compared to untreated diabetic rats and diabetic rats treated with glibenclamide ($p<0.05$). KP at 420 mg/kg improved the epididymis and seminal vesicle weights, which were comparable to those in normal nondiabetic rats.

The serum testosterone level is presented in figure 1. Serum testosterone level in diabetic rats was significantly different from that in normal rats, whereas treatment with KP significantly increased serum testosterone level in diabetic rats ($p<0.05$).

Table 1 Effect of *K. parviflora* extract on blood glucose

Groups	Blood glucose (mg/dL)	
	Before treatment	After treatment
Group 1	101.75±4.59 ^a	102.75±4.78 ^a
Group 2	305.25±27.01 ^b	403.25±36.77 ^b
Group 3	287.25±40.80 ^b	420.00±31.77 ^b
Group 4	279.75±56.37 ^b	425.75±14.17 ^b
Group 5	305.00±23.34 ^b	463.00±47.41 ^b
Group 6	274.60±59.30 ^b	414.60±40.54 ^b

All data are shown as the mean±SEM,

Value in each row marked different superscript letter differ significantly ($p<0.05$)

Table 2 Effect of *K. parviflora* on testis, epididymis and seminal vesicle weight

Groups	Reproductive organ weight (g)		
	Testes	Epididymis	Seminal vesicle
Group 1	3.65±0.12 ^a	1.36±0.16 ^a	1.13±0.21 ^a
Group 2	2.14±0.53 ^b	0.71±0.16 ^b	0.26±0.11 ^b
Group 3	3.05±0.27 ^b	0.96±0.16 ^b	0.67±0.26 ^b
Group 4	3.19±0.07 ^a	0.99±0.11 ^{ab}	0.59±0.22 ^{ab}
Group 5	3.23±0.08 ^a	1.06±0.11 ^{ab}	0.66±0.10 ^{ab}
Group 6	3.65±0.15 ^a	1.26±0.09 ^a	0.95±0.28 ^a

All data are shown as the mean±SEM,

Value in each row marked different superscript letter differ significantly ($p<0.05$)



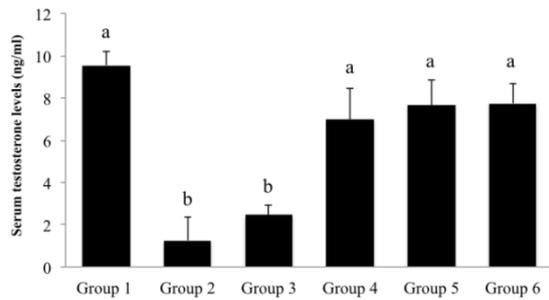


Figure 1. Effects of *K. parviflora* on serum testosterone levels. Data are presented as the mean±SEM. The different characters indicated significant differences ($p<0.05$).

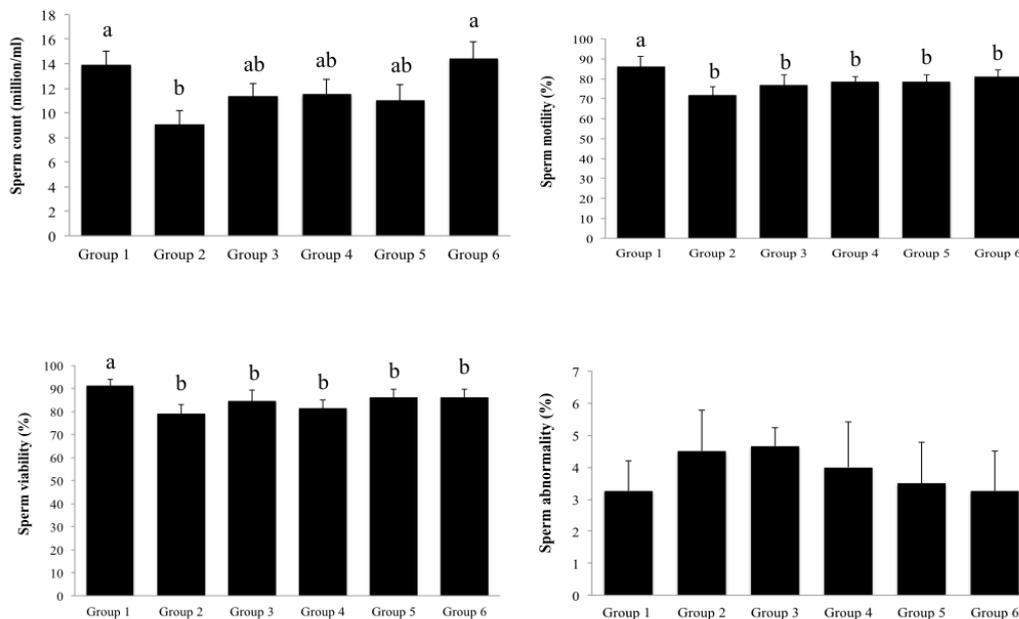


Figure 2. Effects of *K. parviflora* on epididymal sperm parameter. Data are presented as the mean±SEM. The different characters indicated significant differences ($p<0.05$).

Effect of KP on Johnson's score and testicular histological structure

DM control rats revealed a significant decrease in the mean Johnson score in comparison with the normal control group (Table 3). Treatment with KP increased the Johnson

Effect of KP treatment on epididymal sperm parameter

The sperm count, motility and viability (Figure 2) were significantly reduced in diabetic rats and treatment with KP at 420 mg/kg revealed significant increases in sperm count ($p<0.05$). However, KP treatment does not have effect on sperm motility and viability compared to diabetic rats. There were no significant differences in the percentage of abnormal sperm between normal and diabetic rats.



tubule diameter that was not statistically significant as compared with the normal rats.

Table 3 Effect of *K. parviflora* extract on Johnson's score and seminiferous tubule diameter

Groups	Johnson's score	Seminiferous tubule diameter (μm)
Group 1	9.63 \pm 0.09 ^a	257.29 \pm 3.09 ^a
Group 2	5.54 \pm 0.18 ^b	190.38 \pm 2.15 ^b
Group 3	7.31 \pm 0.17 ^c	230.38 \pm 2.97 ^c
Group 4	7.71 \pm 0.14 ^{c,d}	236.85 \pm 2.94 ^c
Group 5	8.06 \pm 0.12 ^d	254.81 \pm 2.69 ^a
Group 6	9.23 \pm 0.12 ^a	261.21 \pm 2.65 ^a

All data are shown as the mean \pm SEM

Value in each row marked different superscript letter differ significantly ($p < 0.05$)

The normal rats exhibited normal testicular structures of the seminiferous tubules, interstitial structure and spermatogenesis (Figure 3 and 4). The diabetic groups manifested with degenerate seminiferous tubules with remarkable reduction in the number of germ cells (Spermatogonia, Spermatocyte, Spermatid and Sertoli cells) and the premature detachment of germ cells. The administration of KP to diabetic rats improved the testicular structure and increased the germinal epithelium thickness and numbers of Spermatogenic cells.

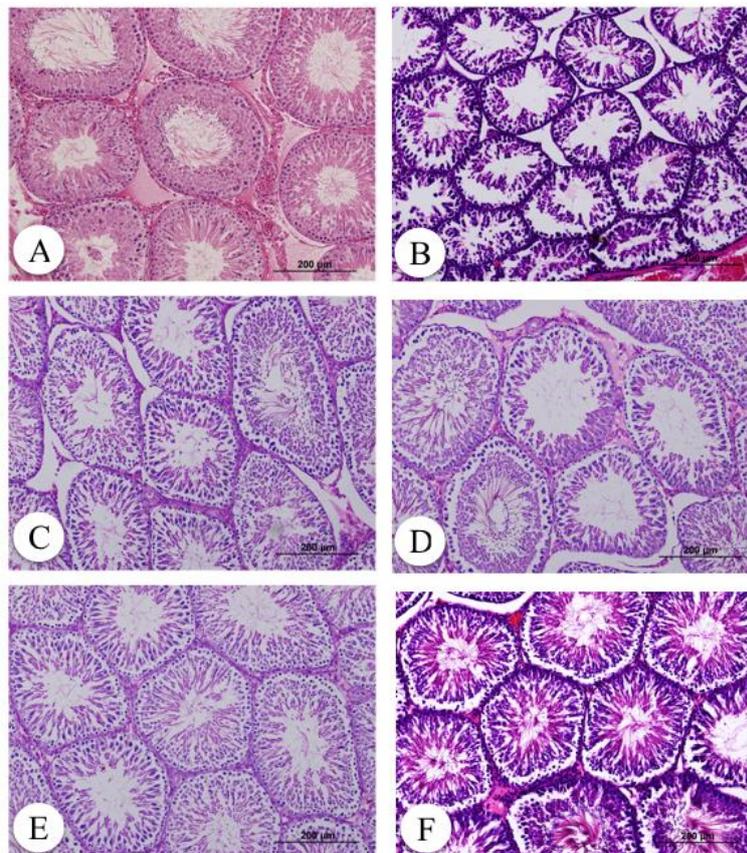


Figure 3. Light photomicrograph of rats' testicular seminiferous tubules stained with H&E (magnification: x100). Group 1 (A), Group 2 (B), Group 3 (C), Group 4 (D), Group 5 (E) and Group 6 (F).

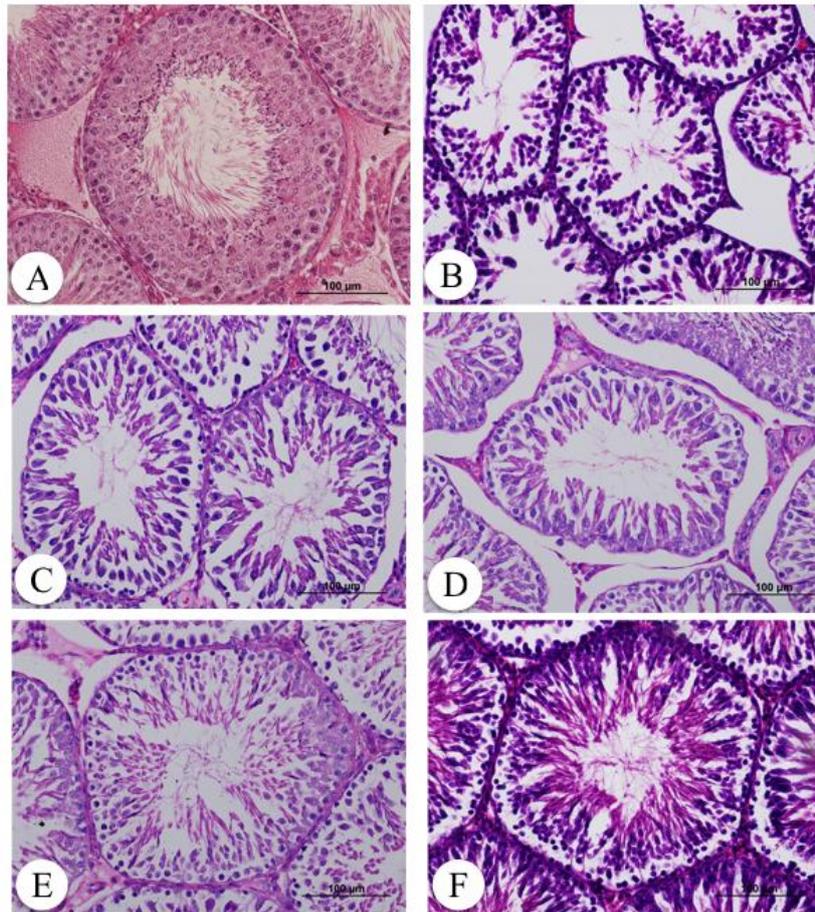


Figure 4. Light photomicrograph of rats' testicular seminiferous tubules stained with H&E (magnification: x400). Group 1 (A), Group 2 (B), Group 3 (C), Group 4 (D), Group 5 (E) and Group 6 (F).

Discussion

The present study clearly reveals that, KP administration in STZ-induced diabetic rats significantly increased testicular weight, sperm concentration, serum testosterone levels and ameliorated testicular structure.

Sexual dysfunctions are frequently associated with diabetic patients and diabetic-induced animals (Bayram et al., 2015; Scarano et al., 2006). The STZ-induced diabetic rat model has been used for evaluation of the effects of diabetes in male reproductive function. The

previous study reported that STZ-induced diabetes in the animal model causes abnormal histology and atrophy of the seminiferous tubules, reduced number of spermatogenic cell series spermatogenic dysfunction, teratozoospermia, testicular dysfunction, increase apoptotic cell deaths in spermatogenic series and decreased serum testosterone levels (Navarro-Casado et al., 2010; Shrilatha, 2007). The present study clearly confirms the disruption of spermatogenesis, significantly decrease sperm density, sperm motility, reproductive organ weight, Johnson's score and seminiferous tubule diameter. It has



been suggested that oxidative stress is a major cause of testicular dysfunction in STZ-induced diabetic animals or an imbalance in the oxidant defense system (Atalay et al., 2012; Shrilatha, 2007; Xu et al., 2014).

The present study demonstrated that the KP treatment in DM rats could improve the serum testosterone levels, testicular weight, sperm density and testicular structure. Furthermore, the aphrodisiac properties of KP in the present report are similar to those of the previous studies, by enhancing sexual performance and serum testosterone levels. In addition, it is shown that immature rats treated with KP could improve their serum testosterone levels with no effect on the reproductive organs (Trisomboon et al., 2007). KP could also increase ejaculation volume, sperm viability, progressive sperm motility, seminal vesicular weight and spermatogenesis both in rabbits and rats (Jitjaingam et al., 2005; Somphol et al., 2003). The histological studies indicate abatement of Leydig cells and disruption of the seminiferous tubules in nontreated and glibenclamide treated diabetic rats but no damage in the Leydig cells of KP administration rats. Hence, it is quite clear that testosterone level is related with the damage of cells. Testosterone has been reported to play a role in growth of male reproductive organs, accessory sex organs and sexual activity (De et al., 2016). Our results indicated that KP treatment in STZ-induced diabetes rats could improve testicular weight with the highest dose showing significantly increase epididymis and

seminal vesicle weight. However, the mechanisms of KP on the androgenic activity in the male rats remain unknown. It is suggested that the increase in spermatid blood flow to the testis stimulates testosterone production and secretion (Chaturapanich et al., 2008). It was shown that flavonoid and phenols, also present in KP extract, induced vasodilation in the corpus cavernosum, and suggesting that the flavonoid may increase the testosterone levels (Wankeu-Nya et al., 2014). Previous reports have demonstrated that KP contains many flavonoids such as 3,5,7,3',4'-pentamethoxyflavone (PMF) causing relaxation of the human corpus cavernosum (Jansakul et al., 2012) and 5,7-dimethoxyflavone (DMF) having a phosphodiesterase5 (PDE5) inhibitor effect (Temkitthawon et al., 2011). DMF enhances testosterone production via cAMP in mouse testis-derived tumor cells (Horigome et al., 2016). The other experiment has demonstrated that KP extract, DMF and 5-hydroxy-3,7,3',4'-tetramethoxyflavone (TMF) reducing intracellular ROS production in human umbilical vein endothelial cells (Horigome et al., 2015).

It was concluded that KP treatment attenuated diabetes related testicular dysfunction, enhanced serum testosterone levels and ameliorated testicular microstructure in STZ-induced diabetes male rats. Further study is undertaken to assess the effects of bioactive compounds of KP on steroidogenesis and sexual behavior in STZ-induced diabetic rats.



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