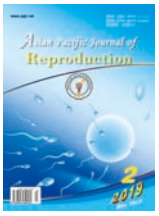

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Effect of combination of *Gynura procumbens* aqueous extract and *Trigona* spp. honey on fertility and libido of streptozotocin-induced hyperglycaemic male rats

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ABSTRACT

Objective: To study the effects of co-administration of *Gynura procumbens* (GP) and kelulut honey (KH) on male fertility and libido in diabetes-induced rats.

Methods: A total 42 males Sprague Dawley rats aged 8 weeks were randomly and equally divided into six different groups. All groups except a normal control group were induced with 50 mg/kg of streptozotocin (STZ) intravenously to induce diabetes. A positive control group was treated with an antidiabetic drug, metformin (500 mg/kg) whereas a negative control group remained untreated throughout the experiment. Meanwhile, another three treatments on diabetic rat groups were performed and categorised as Group 1 (450 mg/kg GP + 300 mg/kg KH), Group 2 (450 mg/kg GP + 600 mg/kg KH) and Group 3 (450 mg/kg GP + 1 200 mg/kg KH). Treatments were given for seven consecutive days through oral gavage and all rats were euthanized on day 8th for fasting blood glucose analysis, sperm quality, spermatogenesis, sexual behaviour and implantation sites analysis.

Results: Fasting blood glucose levels were significantly reduced after treatment of GP and KH, compared to negative and positive controls. The treated groups showed significant increment in sperm quality compared to all control groups. Testes histology illustrated significant damages on leydig and sertoli cells for both negative and positive controls. On the contrary, co-administration of GP and KH displayed regeneration of leydig and sertoli cells in the testes. Additionally, the number of implantation sites significantly increased in females copulated with treated groups, compared to controls. Besides, the libido analysis displayed improvement of libido in treated groups, compared to all controls. Throughout the study, insignificant variances were recorded between the treated groups, indicating that treatment in Group 1 was sufficient to trigger significant improvement on fasting blood glucose level, fertility, and libido in diabetic male rats.

Conclusions: Co-administration of GP and KH has great potential to serve as a pro-fertility agent amongst diabetic patients.

1. Introduction

Diabetes mellitus has been long associated with reproductive impairment, particularly in male reproduction health. Studies have shown that diabetic patients experience several side effects or dysfunction in fertility, such as low sexual desire, erectile dysfunction, and difficulties in producing offspring[1]. According to La Vignera *et al*[2], diabetes-induced rats exhibited low sperm quality and interrupted spermatogenesis in the testes. This statement is supported in the finding of Jangir and Jain[3], where diabetes mellitus caused hypospermatogenesis, decreased percentage of sperm motility and normal sperm morphology. Studies showed that

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diabetes mellitus interrupts the production of lactate which is the fuel for sperm development[4]. This event caused alteration in seminal parameters, and hence resulted in poor sperm morphology[5]. According to the meta-data compiled by Vasilios *et al*[6], the event of diabetes mellitus affected the total seminal volume and the percentage of motile sperm.

Diabetes mellitus refers to a metabolic disorder that is characterized by several syndromes, such as hyperglycaemia, altered lipid, carbohydrate and protein metabolism, as well as increased risk of vascular diseases[7].

Conventional medicines, such as glibenclamide or metformin, have been established into reducing blood glucose level in diabetic patients. Nevertheless, these drugs have yet to show sign of improving fertility caused by diabetes mellitus[8]. Aside from the inability to improve fertility, these drugs have been reported to give long-term side effects, such as nausea, stomach pain, and diarrhoea.

Herbs and medicinal plants have been long used in traditional practices due to their benefits and therapeutic effects in treating illnesses. Herbs, such as *Tongkat ali*, *Kacip fatimah* and *Tunjuk langit*, have been studied in order to explore their potentials as alternative medicines in treating diseases. Even the government seems to place focus onto herbs due to their purity and efficacy in terms of pharmacological effects[9]. *Sambung nyawa* is a medicinal plant that has been determined to address various ailments. This herb, which is scientifically known as *Gynura procumbens* (GP), is a shrub plant that widely grows within the Southeast Asia region[10]. This herb has displayed potential effect as anti-hyperglycaemic[11], anti-inflammatory[12], anti-hypertensive[13], and recently, as a pro-fertility agent[14]. GP extract also appears to be a potent source of antioxidant due to its phenolic content[15].

A number of studies have investigated the combination of herbs in treating various types of infirmities. Khaki *et al*[16] reported that the combination of cinnamon and ginger could improve fertility in diabetic male rats. Another study highlighted that the combination of GP and *Andrographis paniculata* displayed a significant reduction of fasting blood glucose (FBG) level in diabetic male rats. As such, this study looked into the combination of GP extract and kelulut honey (KH)[17].

Honey is part of traditional medicine that has been long used in treating ailments, such as healing wounds and burns. The KH is produced by the 'kelulut' bees, which are known as *Trigona* spp. These stingless bees can be found in Malaysia. The honey produced by *Trigona* spp. is multifloral honey stored in clusters of small resin pots. Unlike honeys from *Apis* sp., which is stored in hexagonal-shaped honey combs, KH has distinct taste and aroma, as well as more fluidity in its texture[18]. Honey is rich in phenolic compound that correlates with antioxidant activity[19]. Kek *et al*[20] reported that KH from *Trigona* spp. contains high level of phenolic compound, which is associated to antioxidant. Honey has also been reported to have fertility effect by enhancing spermatogenesis in rats[21]. Liquid chromatography-mass spectrometry analysis conducted by Yazan *et al*[22] reported that several flavonoids were found in KH that played essential roles in anti-hyperglycaemia and fertility health, such as quercetin and kaempferol.

The prior studies showed that both GP extract and KH could improve fertility in male rats[14,21]. Nonetheless, no study has evaluated the combination effect of both GP extract and KH on fertility among diabetics condition. Therefore, this study was to examine the synergistic effect of GP extract co-administered with KH on blood glucose level, libido and fertility, in terms of sperm quality, spermatogenesis, sexual behaviour as well as implantation sites in diabetes-induced male rats.

2. Materials and methods

2.1. Preparation of GP and KH

For the purpose of this study, only the leaves of GP were used. The herbs were collected from glass house located at Universiti Kebangsaan Malaysia. GP sample was deposited to the Herbarium in Faculty of Science and Technology, Universiti Kebangsaan Malaysia (UKM) with voucher number 40343 [KAK 01 (UKMB)]. The GP leaves were oven-dried for 72 h at 48 ℃. The dried leaves were ground to powder form and extracted using distilled water as the solvent/medium. Next, the aqueous extract of GP was freeze-dried and kept at 4 ℃ to retain its freshness until further analysis.

KH was collected from Universiti Teknologi MARA located at Puncak Perdana. The sample was weighed by using a weighing balance and prepared in three different dosages: 300, 600, and 1 200 mg/kg KH. The prepared doses were co-administered with GP extract (450 mg/kg GP), respectively.

2.2. Animal husbandry

This study was approved by the Animal Ethics Committee of Faculty of Medicine, Universiti Kebangsaan Malaysia (FST/2013/MAHANEM/31-JAN./492-FEB.-2013-FEB.-2015). The rats were kept in polyvinylchloride cages at controlled room temperature with 12 h light-12 h dark cycle. The male rats were acclimatized for seven days, wherein their food and drink intakes were given *ad libitum*. All animal subjects were proven fertile prior experiment and provided by the Animal House of Universiti Kebangsaan Malaysia.

A total of 42 male Sprague Dawley rats aged 8 weeks were used in this study. For this study, all rats were divided randomly and equally into six different groups. Three groups were categorized as treatment groups: Group 1 (450 mg/kg GP + 300 mg/kg KH), Group 2 (450 mg/kg GP + 600 mg/kg KH) and Group 3 (450 mg/kg + 1 200 mg/kg KH), while another three groups were categorized as the controls: normal, positive, and negative controls. All groups, except the normal control group, were induced with 50 mg/kg of streptozotocin (STZ). The positive control group was treated with metformin (500 mg/kg), while the negative control group remained untreated throughout the experiment. Treatment was given *via* oral gavage for seven consecutive days. On the 8th day, all rats were euthanized for further fertility analysis. Treatment was given for seven consecutive days to determine the time for the combination extract starts to affect FBG level and other parameters. This duration was chosen since the effect was proven by previous study[14], whereby the experiment showed significant results after 14 days of treatment.

2.3. Induction of STZ

STZ was prepared in citrate buffer, pH 4.5 at the dose of 50 mg/kg per rat bodyweight. All animal subjects except the normal control group were induced with STZ. The STZ was injected intravenously at the root of the rats' tail after an overnight fasting. FBG level was measured after seven days of induction. Blood glucose level that exceeded 13 mmol/L was considered as diabetic.

2.4. FBG level analysis

After treatment of seven days, FBG level was measured by using a glucometer Accucheck perfoma. Blood was collected from the tip of the tail with a glucose strip and the FBG level was determined.

2.5. Sperm quality analysis

The rats were sacrificed and the caudal epididymises were removed to obtain sperm samples. The caudal epididymises were minced and suspended in the Biggers-Whitten-Whittingham medium[23]. The samples were then incubated in 5% CO_2 incubator for 30 min at 37 ℃ to allow the sperms to swim up. The sperm sample quality was assessed based on the following parameters: sperm count, motility, and viability. The sperm samples were assessed by adhering to the World Health Organization[24].

2.6. Spermatogenesis study

Spermatogenesis study was performed *via* quantitative and qualitative analyses. The testes were removed and fixed in a Bouins solution for overnight. Later, the testes were dehydrated by using series of alcohols, and finally embedded in paraffin. The testes samples were sectioned at 5 μm in thickness and stained using Mallory staining. Testicular histology was observed under light microscope. Quantitative analysis was performed by measuring the thickness of germ cell on testicular histology of all groups.

2.7. Sexual behaviour study

Sexual behaviour test was performed to determine the effect of the treatment on rats after seven days of treatment. The test was performed based on the rats' sexual protocol proposed by Agmo[25]. A total of 84 healthy female rats were used in this study and made receptive for sexual activity test. A male rat was placed in the test cage for the first ten minutes and then followed by two female rats. The observation period was 15 min. Sexual behaviour was determined by observing mounting frequency and mounting latency. Mounting frequency was the amount of mounting that occurred within 15 min observation, meanwhile, mounting latency was the time taken for the first mount to occur within 15 min observation.

2.8. Implantation sites

After seven days of treatment, each male rat was kept in a different cage together with two oestrous female rats. The rats were allowed to mate for seven days. Vaginal smears were performed to determine gestation day. Pregnant female rats were later separated and kept for 16 days before they were sacrificed and the number of foetuses in the uterus were recorded[26].

2.9. Statistical analysis

The statistical analysis was performed in SPSS 22.0, using one-way analysis of variance (ANOVA) with a P value <0.05 considered statistically significant. The data were presented as mean ± standard error of means (mean ± SEM).

3. Results

3.1. FBG level

Based on the results, the administration of the GP aqueous extract and KH in combination for seven consecutive days showed a significant reduction of FBG level when compared to both negative and positive control groups (Figure 1). After treatment, FBG level in all treated groups (Group 1, Group 2 and Group 3) was insignificant when compared to that of the normal group. This implied that the co-administration of GP aqueous extract and KH reduced the blood glucose level to the normal level after seven days of treatment (Figure 1). Treatment of metformin in positive control group showed a decrease of FBG level as well. Nevertheless, the decrease was not improved to the normal level of FBG.

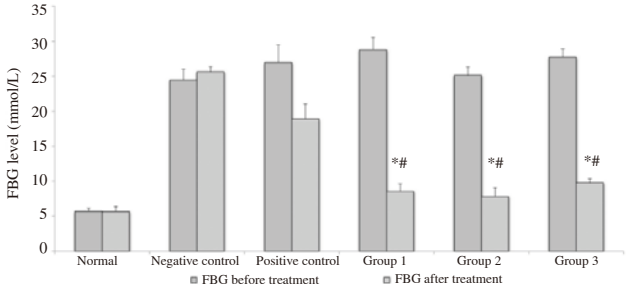

Figure 1. Effects of GP + KH on blood glucose level after seven-day treatment in all groups.

Group 1 (450 mg/kg GP + 300 mg/kg KH), Group 2 (450 mg/kg GP + 600 mg/kg KH) and Group 3 (450 mg/kg GP + 1 200 mg/kg KH) as treatment groups. * Compared to negative control after treatment; # compared to positive control after treatment.

3.2. Sperm quality analysis

The effect of oral administration of GP aqueous extract and KH for seven consecutive days on the sperm quality of diabetic induced male rats was presented in Table 1. Daily administration of treatment showed that the overall sperm quality significantly increased in treated groups when compared to that in negative control group. The sperm count, sperm viability and sperm progressive motility

of treated groups significantly increased, particularly the group treated with 450 mg/kg GP + 1 200 mg/kg (Group 3), as compared to negative control group (Table 1). Nevertheless, treatment of metformin in positive control group displayed nil improvement for sperm quality when compared to treatment groups.

3.3. Spermatogenesis study

Figure 2 presented the outcomes obtained from the qualitative analysis of spermatogenesis for all groups. Testes histology of both negative and positive control groups showed empty lumen with disrupted spermatogenesis. Based on the results, testicular tissue sections in the treated group indicated that the treatment of GP aqueous extract and KH improved testicular impairment, especially the sertoli and leydig cells. The lumen in each treatment group was packed with sperm, hence implying that the combination of GP and KH did improve spermatogenesis.

Quantitatively, the thickness of germ cell of each testis was measured and tabulated in Table 1. The thickness of germ cell in the treated groups, specifically in Group 3, appeared to improve significantly, when compared to both negative and positive control groups.

3.4. Sexual behaviour study

The mounting frequency of all groups was presented in Table 2. Treatment groups illustrated a significant increment of mounting frequency, when compared to both negative and positive control groups. Table 2 showed that the mounting frequency in Group 3 significantly increased, when compared to all the other groups. The negative control group exhibited the lowest number of mounting frequency; whereas the positive control group displayed a low number of mounting frequency. The statistical analysis demonstrated insignificant variances between the treated groups.

Table 1. Effects of GP + KH on sperm parameters and germ cell thickness in testes after seven-day treatment in all groups.

Groups	Sperm count ($\times 10^6$)	Sperm viability (%)	Sperm progressive motility (%)	Germ cell thickness (μm)
Normal	$36.47 \pm 1.42^{b,c}$	58.26 ± 2.06	$30.46 \pm 2.12^{b,c}$	$75.87 \pm 2.12^{b,c}$
Negative control	21.75 ± 1.30	57.49 ± 3.30	16.96 ± 5.13	54.95 ± 1.19
Positive control	21.43 ± 0.71	58.68 ± 1.75	21.75 ± 1.99	56.74 ± 4.91
Group 1	$41.11 \pm 2.33^{a,b,c}$	$74.58 \pm 3.06^{a,b,c}$	$46.38 \pm 3.67^{a,b,c}$	$68.40 \pm 2.52^{a,b,c}$
Group 2	$40.41 \pm 1.83^{a,b,c}$	$75.89 \pm 1.89^{a,b,c}$	$48.75 \pm 2.70^{a,b,c}$	$72.00 \pm 3.52^{a,b,c}$
Group 3	$41.50 \pm 2.28^{a,b,c}$	$82.36 \pm 1.35^{a,b,c,*}$	$52.75 \pm 3.13^{a,b,c,*}$	$75.52 \pm 3.76^{a,b,c,*}$

[a] significant compared to normal group ($P<0.05$); [b] significant compared to negative control group ($P<0.05$); [c] significant compared to positive control group ($P<0.05$). *: significant compared between Group 1 and Group 3 ($P<0.05$). Group 1: 450 mg/kg GP + 300 mg/kg KH; Group 2: 450 mg/kg GP + 600 mg/kg KH; Group 3: 450 mg/kg GP + 1 200 mg/kg KH.

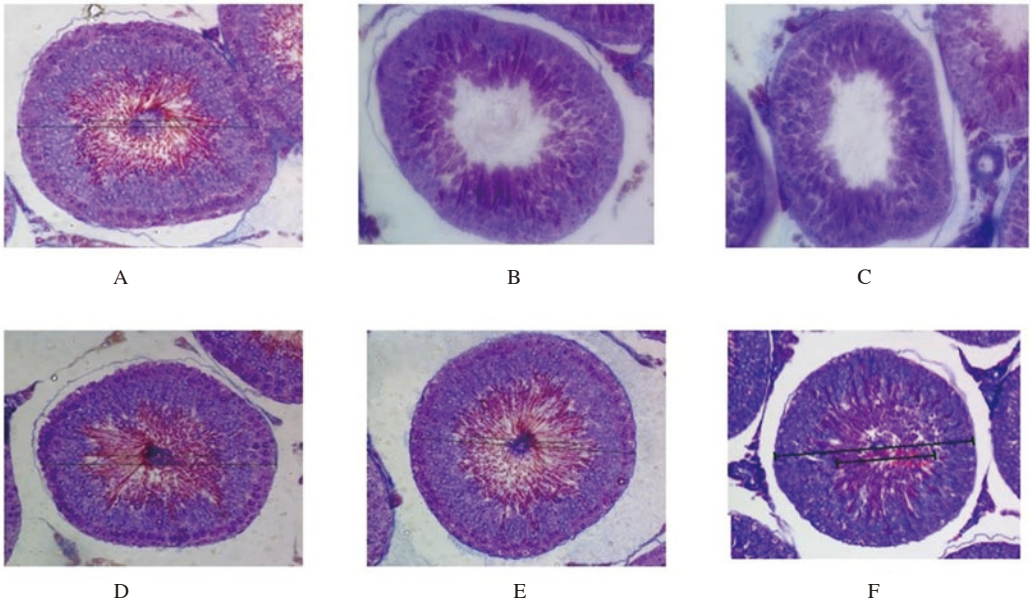

Figure 2. Effects of GP + KH on testes histology after seven-day treatment (400 × magnification; Mallory staining).

(A) Normal, (B) Negative control, (C) Positive control, (D) Group 1 (450 mg/kg GP + 300 mg/kg KH), (E) Group 2 (450 mg/kg GP + 600 mg/kg KH), (F) Group 3 (450 mg/kg GP + 1 200 mg/kg KH). Testes histology of both negative and positive control groups showed empty lumen with disrupted spermatogenesis. The treated groups demonstrated that the combination treatment of GP aqueous extract and KH improved testicular impairment.

In this study, the results for mounting latency of all groups was presented in Table 2. Based on the results attained, the mounting latency for the negative control showed the longest time taken for the first mount to occur. Interestingly, treatment of GP aqueous extract and KH in combination illustrated a significant mounting latency compared to other groups, whereas treatment using metformin showed shorter amount of time compared to negative control. However, the mounting latency was insignificant compared to all treatment groups.

Table 2. Effects of GP + KH on sexual behaviour (libido) after seven-day treatment in all groups.

Groups	Mounting frequency	Mounting latency (min)
Normal	8.00 ± 0.78[b,c]	7.43 ± 0.61[b,c]
Negative control	1.00 ± 0.46	14.14 ± 0.46
Positive control	1.00 ± 0.55	13.86 ± 0.70
Group 1	9.00 ± 0.89[b,c]	7.43 ± 0.75[b,c]
Group 2	10.00 ± 0.52[a,b,c]	6.00 ± 0.58[b,c]
Group 3	11.00 ± 0.29[a,b,c]	6.57 ± 0.43[b,c]

[a] significant compared to normal group (P<0.05); [b] significant compared to negative control group (P<0.05); [c] significant compared to positive control group (P<0.05). Group 1: 450 mg/kg GP + 300 mg/kg KH; Group 2: 450 mg/kg GP + 600 mg/kg KH; Group 3: 450 mg/kg GP + 1 200 mg/kg KH.

3.5. Implantation sites

Implantation sites were counted after the fertility test was performed. Based on the outputs, all treated groups demonstrated a higher number of implantation sites, when compared to negative (1.00±0.37) and positive (2.00±0.57) control groups. The administration of the combination showcased a significant increment in the number of implantation sites upon copulation with oestrous female rats. The group treated with 450 mg/kg GP + 300 mg/kg KH (Group 1) recorded 9.00±1.02 implantation sites, while Group 2 obtained 10.00 ±0.74, and Group 3 had 11.00±0.57 implantation sites. The number of implantation sites of all treated groups were found to be insignificant compared to the normal control (10.29±1.02). This signified that co-administration of GP aqueous extract and KH did improve both fertility and sperm functionality in diabetic-induced male rats.

4. Discussion

Diabetes mellitus causes drastic incline of blood glucose level that may result in hyperglycaemic condition. The rising number of diabetic patients, especially at reproductive age, has raised concerns among researchers. Synthetic drugs have been commonly used in conventional medication to reduce the level of blood glucose in patients. Nonetheless, with a long list of side effects caused by these drugs, researchers have shifted the attention to medicinal plants as the alternatives in treating diseases.

It is noteworthy that diabetes mellitus has defecting consequences on male reproductive health[16]. This disease can cause testicular impairment[21], low sperm count[8], and increment in reactive oxygen species[27]. The present study revealed that diabetes has significant deleterious effects on sperm quality (count, viability, and motility) and impaired spermatogenesis. This is consistent with the destructive findings reported in past studies pertaining to diabetic subjects[2,8,11,14,28]. From the outcomes, STZ-induced diabetic male rats in the negative control group displayed a significant elevation in blood glucose level. This result concurs with that reported by Hassan *et al*[29]. Co-administration of GP and KH for seven days had significantly reduced FBG level of diabetes-induced male rats. GP has been reported to have the ability to mimic insulin[29]. *In vitro* study showed that supplementation of GP aqueous extract increased the intake of glucose in peripheral muscle that mimics the action of insulin in reducing the level of blood glucose in the bloodstream[29]. The combination of GP and KH showed significant reduction of blood glucose level after only seven days of treatment. Furthermore, treatment of GP co-administered with KH displayed significant ameliorating effects on sperm quality and on damaged spermatogenesis. To the best of our knowledge, this study is the first to reveal the beneficial synergistic effects of GP and KH on FBG level, fertility, and libido in diabetic rats.

Prolonged state of hyperglycaemia in diabetes mellitus can lead to the formation of free radicals. Since free radicals have the ability to interact with nucleic acids, lipids and proteins, they may generate an oxidative stress condition, hence causing destructive effects[30]. The generated oxidative stress has been reported to affect the male reproductive function by disrupting steroidogenesis and altering the germinal epithelium ability to differentiate spermatozoa[31]. Oxidative stress can cause sperm metabolism impairment and deteriorate sperm quality[32]. Mammalian testis and sperm cells are vulnerable to oxidative stress since they are highly made up of polyunsaturated fatty acids, thus putting sperm and testis at risk of oxidative damage[8,33]. In fact, a study reported that the sperms of diabetic male patients were more susceptible to DNA damage, apart from experiencing low sperm quality due to oxidative effect[34].

Herbal medicine and supplementation of natural antioxidants have been used to treat diabetes and several other infirmities since ancient times[35]. Inevitably, herbal plants, such as ginger, cinnamon, and GP, as well as natural antioxidants like honey, owe their therapeutic effects to their antioxidant properties and their polyphenol content, which have been reported to be effective in treating various ailments, such as hyperglycaemia and infertility[16,36,37]. Moreover, GP and KH both have been found to improve sperm quality, testicular function, and androgen hormones[11,14,21]. This study revealed that sperm quality, spermatogenesis, and libido can be substantially enhanced following treatment with GP extract and KH. Besides, the number of implantation sites increased significantly after treatment. Although several studies displayed similar outcomes, the effects of GP extract[8,14] and KH[21] were investigated separately on fertility in diabetic male rats. The presence of antioxidants in GP extract have been reported to be responsible in addressing the overproduction of reactive oxygen species in diabetes-induced male rats[15]. Honey

is known for its phenol content and its antioxidant properties. Compared to honey produced by *Apis* sp., and honey generated by *Trigona* spp. possesses higher phenolic and antioxidant contents[20].

Metformin is an established drug used to control the level of blood glucose in diabetic patients. However, this drug has been reported only to be effective in reducing blood glucose level, but not to enhance fertility or libido of the subjects. In this study, the administration of metformin in the positive control group had managed to decrease the level of FBG although the reduction was insignificant when compared to the negative control group. Consumption of metformin in the positive control group also revealed that sperm quality, spermatogenesis, libido, and implantation sites did not improve after the seven-day treatment. This finding is parallel to that of the previous investigations[8,11,14].

High blood glucose induces changes in leydig cells, including alteration in the hypothalamic-pituitary-gonadal axis, and hence causes deterioration in androgen synthesis[38]. This study shows that diabetic rats from the negative control group had testicular impairment with degenerated leydig and sertoli cells. Formation of alien vacuoles was noted in the testis histology of diabetic rats. Spermatogenesis refers to sperm development process that is regulated by the androgen hormones. Luteinizing hormone is responsible to form normal leydig cell function[39] and plays a bigger role in producing testosterone[40]. The significant improvement in the testes of diabetic rats following GP plus KH treatment in this study had been most probably due to the decrease in blood glucose level, as well as the presence of bioactive compound that possesses antioxidant properties. The improvement of testicular histology in the treatment group resulted in normal production of androgen hormones, hence, improvement in spermatogenesis. This study proves that treatment of GP and KH could improve libido in the diabetic rats and increase the number of implantation sites after mating with oestrous female rats.

To the best of our knowledge, this study is the first to combine GP extract and KH in terms of determining the effect on both hyperglycaemia and male fertility. However, further studies need to be conducted such as using molecular approach. Although sperm quality parameters, like sperm count, motility, and morphology, were found to be the standard analysis to determine male infertility, these conventional methods can be further analysed by using molecular study. According to Vasilios *et al*[6] and Kamaruzaman *et al*[41], through molecular screening, such as DNA or sperm fragmentation as well as sperm proteomic analysis could clarify and reveal the sperm factors that might contribute, leading our understanding in male fertility-infertility issues.

In conclusion, diabetes mellitus and male reproduction dysfunction have long been associated. The increasing number of diabetic patients, especially at reproductive age, suggests the decreasing preponderance of male fertility health. Although several antidiabetic drugs in the market have been proven to reduce the condition of hyperglycaemia amongst diabetic patients, they have no positive effect on diabetes mellitus-related complications and health reproduction. Nevertheless, this study introduces a combination of herbs and honey as an alternative antidiabetic therapy that can control glycaemic level, besides maintaining the male reproductive function. As a conclusion, this study shows that the co-administration of GP extract and KH, when compared with GP and KH alone, in diabetic rats significantly improved the damaging effects of oxidative stress on spermatogenesis and fertility parameters. It seems that the antioxidant contents in GP and KH can be increased drastically when in combination. This study also displays that the treatment is most effective at higher dosage (450 mg/kg GP + 1 200 mg/kg KH), with initial effect noted at the lowest dose of 450 mg/kg GP + 300 mg/kg KH. Therefore, the co-administration of GP and KH has the potential to serve as a plant-based product in treating diabetic patients who suffer from infertility complications.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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