Original Communication

Hypoglycemic and Antioxidant Effects of Honey Supplementation in Streptozotocin-induced Diabetic Rats

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Abstract: *Objectives:* Oxidative stress plays a crucial role in the development of diabetic complications. The aims of this study were to investigate whether honey could reduce hyperglycemia and ameliorate oxidative stress in kidneys of streptozotocin-induced diabetic rats. *Methods:* Diabetes was induced by a single dose of STZ (60 mg/kg; i.p.). Diabetic rats were randomly grouped and administered distilled water (0.5 mL/day) and honey (0.2 g/kg/day, 1.2 g/kg/day and 2.4 g/kg/day) by oral gavage for four weeks. Each group consisted of six rats. *Results:* Total antioxidant status (TAS), activities of catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) were significantly reduced, while superoxide dismutase (SOD) activity was up-regulated in kidneys of diabetic rats. Lipid peroxidation (TBARS) and fasting plasma glucose (FPG) were significantly elevated while body weight was reduced in diabetic rats. Honey significantly increased body weight, TAS, activities of CAT, GPx, GR, and GST in diabetic rats. It significantly restored SOD activity, and reduced FPG and TBARS levels in diabetic rats. Histopathological examinations of the kidneys revealed that mesangial matrix expansion and thickening of glomerular basement membrane were reduced in the honey-treated diabetic rats. *Conclusions:* Honey exerts a hypoglycemic effect and ameliorates oxidative stress in kidneys of streptozotocin-induced diabetic rats.

Key words: Diabetes mellitus; oxidative stress; antioxidant enzymes; streptozotocin; honey

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Introduction

Diabetes mellitus is characterized by chronic hyperglycemia and development of microvascular complications such as nephropathy, retinopathy, and neuropathy [1]. As a result of its microvascular pathology, diabetes is a leading cause of nephropathy leading to end-stage renal disease (ESRD), which accounts for 35 % of all new cases requiring dialysis therapy in developed countries [2]. Therefore, the importance of preventing the development and progression of diabetic nephropathy cannot be over-emphasized. Oxidative stress, mediated through hyperglycemia, is known to play a crucial role in the development of diabetic complications such as nephropathy [3].

Although optimal control of blood glucose is effective in reducing microvascular complications of diabetes, even optimal control of blood glucose does not prevent oxidative stress-induced diabetic complications [4]. This suggests that alternative treatment strategies are required to prevent diabetic complications. Under physiological conditions, the body is fully protected from the adverse effects of free radicals by a network of the antioxidant defense system [5]. This system becomes impaired in diabetes and it is further exacerbated due to persistent challenge by reactive oxygen species (ROS) generated by hyperglycemia [6]. This often leads to oxidative stress, which is an imbalance of oxidants/antioxidants in favor of the former [7]. The efficiency of this defense mechanism is compromised in diabetes and therefore, the ineffective scavenging of free radicals leads to tissue and organ damage [8]. Oxidative stress is linked to diabetic complications such as nephropathy, retinopathy, neuropathy, and atherosclerotic vascular disease. Antioxidants such as α-lipoic acid, and vitamins C and E have been considered as potential treatment for these complications [9].

Honey is known to be rich in both enzymatic and non-enzymatic antioxidants such as catalase, flavonoids, alkaloids, polyphenols, carotenoid, Maillard-reaction products, and vitamins [10–12]. Honey, as a functional food, has the potential to enhance health or reduce risk of disease. Although the health benefits of honey have been documented, based on published literature, there is no evidence-based experimental study showing the effects of honey on hyperglycemia and oxidative stress in diabetes mellitus. Hence, this present study investigated whether tualang honey (AgroMas®, Malaysia) could reduce hyperglycemia and ameliorate oxidative stress in streptozotocin-induced diabetic rats. Tualang honey is produced by wild Asian bees (*Apis dorsata)*. It gets its name from the tualang tree (*Koompasia excelsa*) that houses the hives of the bees. The tualang tree is a magnificent tree that can be found growing in the Southeast Asian rainforests of peninsular Malaysia, southern Thailand, northeastern Sumatra, Borneo, and Palawan.

Materials and methods

Materials

Chemicals

Streptozotocin (STZ), thiobarbituric acid (TBA), and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other chemicals and reagents used were of analytical grade.

Tualang honey

Tualang honey (AgroMas®, Malaysia) was supplied by the Federal Agricultural Marketing Authority (FAMA), Kedah, Malaysia. It is a pure multi-flora wild honey. The honey was previously evaporated at 40°C to standardize its water content of 20 %.

Honey preparation for animal treatment

The honey was diluted with distilled water and prepared freshly each time it was administered. The doses were worked out relative to local human consumption as supplementation, which is 0.2 g/kg body weight once daily. The honey was administered using an oral cannula.

Animals

Male Sprague-Dawley rats aged 10–12 weeks (250–300 g) were used in this study. The animals were bred in the Laboratory Animal Research Unit of Universiti Sains Malaysia, Health Campus, Kelantan, Malaysia. The rats were acclimatized to the animal room conditions for at least one week prior to the experiment. Ethical approval was obtained from the Animal Ethics Committee of Universiti Sains Malaysia, Malaysia. Institutional Guidelines for the Care and Use of Animals for Scientific Purposes from the Helsinki Declaration regarding animal experimentation were followed. The rats were caged individually in polypropylene cages in a well-ventilated animal

room at ambient temperature of 25±2°C with a 12-hour light/dark cycle. Rats were allowed free access to standard laboratory chow and drinking water *ad libitum*, unless otherwise stated.

Methods

Induction of diabetes and treatment

The animals were fasted overnight for at least 16 hours before induction of diabetes. Diabetes was induced by a single intraperitoneal injection of freshly prepared solution of STZ (60 mg/kg body weight) in 0.1M sodium citrate buffer, pH 4.5. Control rats received sodium citrate buffer alone without streptozotocin. Food and water intake were examined daily after STZ administration. Diabetes was confirmed by measuring fasting blood glucose concentration at 48 hours post-STZ injection. Blood samples were collected from the tail vein and blood glucose levels were determined using an Accu-Chek Glucometer (Roche, Germany). Animals with blood glucose concentrations of $\geq$ 12 mmol/L with other symptoms of diabetes mellitus such as polyuria, polydypsia, polyphagia, and weight loss were considered diabetic and used in this study. The diabetic rats were randomly divided into four groups with six rats in each group. The animals were treated by oral gavage once daily between 9:00 am to 11:00 am for 4 weeks as follows:

Group 1: Non-diabetic rats received distilled water (0.5 mL). This group served as normal control.

Group 2: Diabetic rats received distilled water (0.5 mL). This group served as diabetic control.

Group 3: Diabetic rats were administered tualang honey (0.2 g/kg body weight).

Group 4: Diabetic rats were administered tualang honey (1.2 g/kg body weight).

Group 5: Diabetic rats were administered tualang honey (2.4 g/kg body weight).

Fasting blood glucose was measured weekly. At the end of the experimental period, the animals were fasted overnight. They were sacrificed and immediately opened surgically and the kidneys were excised and processed.

Tissue processing

The left kidney was rapidly excised, washed in ice-cold normal saline, blotted, frozen in liquid nitrogen, and stored at −80°C until use. The right kidney was also excised, fixed in 10 % formalin, and dehydrated in ascending grades of ethanol, cleaned in xylene and embedded in paraffin. Sections (5 mm thick) were cut and stained with hematoxylin and eosin (H&E) before the slides were subjected to photomicroscopic observation. Digital images were obtained from a high-resolution digital camera system (Penguin 150CL, Pixera, Los Gatos, CA, USA) linked to a microscope (BX41, Olympus, Tokyo, Japan) and desktop computer (Pentium 4, 2.0 GHz). Images of glomeruli at 100 X magnification were digitized.

Biochemical analyses of kidney homogenate

Frozen kidneys were thawed and homogenized to make 10 % homogenate (w/v) in ice-cold Tris-HCl (0.1 M, pH 7.4) using an ice-chilled glass homogenizing vessel in a homogenizer fitted with Teflon pestle (Glas-Col, USA) at 900 rpm. The homogenate was centrifuged at 1000 X *g* for 10 minutes at 4 °C in a refrigerated centrifuge to remove the nuclear debris. The supernatant obtained was used for the following assays: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), total antioxidant status (TAS), malondialdehyde (MDA), and total protein (TP).

Superoxide dismutase (SOD) assay

Activity of SOD was measured by the method of Sun *et al.* [13] based on inhibition of nitroblue tetrazolium (NBT) reduction, with xanthine-xanthine oxidase used as a superoxide generator. One unit of SOD is defined as the amount of enzyme that inhibits the rate of NBT reduction by 50 %.

Catalase (CAT) assay

CAT activity was determined according to the method of Goth [14]. One unit of CAT was defined as the amount of enzyme that catalyzes the decomposition of 1 μmol of hydrogen peroxide per minute.

Glutathione peroxidase (GPx) assay

GPx activity was determined according to the method of Dogan *et al.* [15] by measuring the decrease in glutathione (GSH) content after incubating the sample in the presence of hydrogen peroxide (H_2O_2) and sodium azide (NaN3). GPx activity was expressed as unit per mg protein based on a molar extinction coefficient of 6.22×10^3 L/mol/cm. One unit of GPx was defined as the amount of enzyme that

catalyzes the oxidation of 1 nmol of NADPH per minute.

Glutathione reductase (GR) assay

GR activity was assayed according to the procedure of Goldberg and Spooner [16] using oxidized glutathione (GSSG) as a substrate. GR activity was expressed as unit per mg protein based on molar extinction coefficient of 6.22×10^3 L/mol/cm. One unit of GR was defined as the amount of enzyme that catalyzes the oxidation of 1 nmol of NADPH per minute.

Glutathione-S-transferase (GST) assay

GST activity was assayed according to the method of Habig *et al.* [17], which is based on the conjugation of glutathione (GSH) to 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate. GST activity was calculated as unit per mg protein based on a molar extinction coefficient of 9.6×10^3 L/mol/cm. One unit of GST was defined as the amount of enzyme that catalyzes the conjugation of 1 nmol of GSH-CDNB per minute.

Lipid peroxidation (LPO) assay

The extent of lipid peroxidation was determined as the concentration of thiobarbituric acid reactive substances (*TBARS*) according to the method of Ohkawa *et al.* [18]. 1, 1, 3, 3-tetra-ethoxypropane (TEP) was used a standard. TBARS concentration was expressed as nmol of malondialdehyde (MDA) per mg protein.

Total antioxidant status (TAS) assay

TAS was determined according to the method of Koracevic *et al.* [19] using uric acid as standard. TAS was expressed as nmol of uric acid per mg protein.

Protein assay

Protein concentration was estimated by using Bio-Rad Protein Assay Kit II according to the method of Bradford [20] using bovine serum albumin (BSA) as a standard.

Statistical Analysis

Statistical analysis was carried out using SPSS 12.0.1. The data were expressed as medians (interquartile range). Groups were compared by the Kruskal-Wallis H test followed by Mann-Whitney *U* test to identify significance of difference between two groups. A p value <0.05 was considered to be statistically significant while n=6.

Table I: Effect of tualang honey on fasting blood glucose, change in body weight, and total food intake in control and STZ-induced diabetic rats

Group	Blood Glucose (mmol/L)		Change in body weight (g/rat)	Total food intake (g/rat)
	Before treatment	After treatment		
Group 1	4.0 (0.2)	3.9 (0.3)	25.0 (4.8)	631 (63)
Group 2	** 16.9 (6.5)	** 20.6 (5.4)	** -32.0 (10.8)	** 991 (102)
Group 3	** 15.5 (5.3)	** 15.0 (2.2)	**,† -6.0 (27.3)	** 919 (102)
Group 4	** 17.2 (5.0)	**, † 14.0 (2.4)	††, ‡ 24.5 (9.5)	** 904 (104)
Group 5	** 17.0 (2.9)	**, † 13.5 (2.3)	††, ‡ 22.0 (25.0)	** 934 (88)

Table I shows the effect of tualang honey on fasting blood glucose, change in body weight, and total food intake in non-diabetic control rats (Group 1), diabetic control rats (Group 2), and honey-treated diabetic rats: Group 3 (0.2 g/kg), Group 4 (1.2 g/kg), and Group 5 (2.4 g/kg).
Results are expressed as median (interquartile range) and n=6 for all groups.
** $p<0.01$ compared to Group 1; † $p<0.05$ & †† $p<0.01$ compared to Group 2; ‡ $p<0.05$ compared to Group 3.

Results

Effect of tualang honey on fasting blood glucose, body weight, and total food intake in control and STZ-induced diabetic rats

Table I summarizes the levels of blood glucose, changes in body weight, and total food intake in non-diabetic control (group 1), untreated diabetic control (group 2), and honey-treated diabetic rats [group 3 (0.2 g/kg), group 4 (1.2 g/kg), and group 5 (2.4 g/kg)]. A significant ($p<0.01$) increase in blood glucose level was observed in group 2 compared with group 1. Groups 4 and 5 showed a significant ($p<0.05$) decrease in blood glucose compared with group 2. Significant ($p<0.01$) decrease in change in body weight was observed in group 2 compared with group 1. The honey-treated groups showed significant improvement in weight gain. The total amount of food intake was significantly ($p<0.01$) increased in all the groups compared with group 1.

Table II: Effect of tualang honey on the activities of antioxidant enzymes, total antioxidant status, and levels of lipid peroxidation in the kidneys of control and STZ-induced diabetic rats

	Group				
	1	2	3	4	5
SOD (U/mg protein)	80.0 (9)	117.5 (17)**	79.5 (9)††	77.5 (15)††	75.0 (4)††
CAT (U/mg protein)	384.9 (22)	155.6 (20)**	298.1 (12)**,††	297.1 (28)**,††	312.0 (18)**,††
GPx (U/mg protein)	810.6 (27)	690.0 (28)**	780.7 (20)**,††	789.6 (21)*,††	801.4 (40)††
GR (U/mg protein)	141.2 (7)	116.7 (12)**	135.7 (5)*,††	134.9 (10)††	140.5 (9)††
GST (U/mg protein)	110.9 (15)	64.6 (11)**	108.3 (8)††	114.0 (23)††	107.0 (12)††
TAS (nmol/mg protein)	1.3 (0.1)	0.7 (0.1)**	0.9 (0.1)**,††	0.9 (0.1)**,†	1.0 (0.2)**,†
MDA (nmol/mg protein)	1.9 (0.1)	2.5 (0.2)**	2.1 (0.1)**,††	1.9 (0.2)*,††	2.0 (0.2)††

Table II shows the effect of tualang honey on the activities of antioxidant enzymes, total antioxidant status, and levels of lipid peroxidation in the kidneys of non-diabetic control rats (Group 1), diabetic control rats (Group 2), and honey-treated diabetic rats: Group 3 (0.2 g/kg), Group 4 (1.2 g/kg), and Group 5 (2.4 g/kg).
Results are expressed as median (interquartile range) and n=6 for all groups.
* $p<0.05$ and ** $p<0.01$ compared to Group 1; † $p<0.05$ and †† $p<0.01$ compared to Group 2

Effect of tualang honey on the activities of antioxidant enzymes, total antioxidant status, and levels of lipid peroxidation in the kidneys of control and STZ-induced diabetic rats

Table II summarizes the activities of antioxidant enzymes, total antioxidant status, and levels of lipid peroxidation in the kidneys of non-diabetic control (group 1), untreated diabetic control (group 2), and honey-treated diabetic rats [group 3 (0.2 g/kg), group 4 (1.2 g/kg), and group 5 (2.4 g/kg)]. Administration of tualang honey significantly ($p<0.01$) decreased the elevated SOD activity. The honey-treated groups showed significant ($p<0.01$) increases in activities of CAT, GPx, GR, and GST compared with group 2. TAS was significantly ($p<0.05$ & $p<0.01$) elevated in the honey-treated groups compared with group 2. Treatment with tualang honey significantly ($p<0.01$) decreased the elevated levels of TBARS.

Histopathological examination of kidneys

Figure 1 shows the hematoxylin and eosin stained sections of rat kidneys.

Discussion

Streptozotocin (STZ) is a drug of choice used to induce type 2 diabetes in animals. This well-established experimental model is usually characterized by insulin deficiency coupled with insulin resistance [21]. STZ at a single dose (60 mg/kg) has been reported to increase blood glucose and decrease body weight in rats [8]. In diabetes mellitus, reduced body weight is a consequence of proteolysis in skeletal muscle and lipolysis in adipose tissue [22]. Treatment with honey produced a significant increase in body weight in diabetic rats. In this present study, the STZ-treated rats were confirmed to be hyperglycemic 48 hours after STZ administration. All these observed diabetic features show that a single intraperitoneal injection of STZ (60 mg/kg body weight) resulted in a reproducible animal model of diabetes mellitus in our experiment. Honey in a dose-dependent response significantly decreased blood glucose levels in diabetic rats compared with untreated diabetic rats. It has been reported also that honey inhalation reduced blood glucose levels in type 2 diabetic patients [23]. Although honey produced a significant reduction in blood glucose concentration among the honey-treated diabetic groups, the levels of blood glucose were still significantly higher than those of the non-diabetic control rats. Thus, it can be implied that honey exerts moderate glycemic control in diabetic rats. In view of the fact that honey contains many constituents including glucose and fructose, the exact mechanism of its anti-hyperglycemic effect is complex and therefore will require further investigation. However, the anti-hyperglycemic effect of natural products such as honey and medicinal plant extracts is generally believed to be dependent upon the degree of islet β-cell destruction [24]. So, tualang honey could have reduced blood glucose through the remnant pancreatic β-cells. In addition, honey may produce its anti-hyperglycemic effect through fructose, which is the most predominant constituent in honey. Fructose has

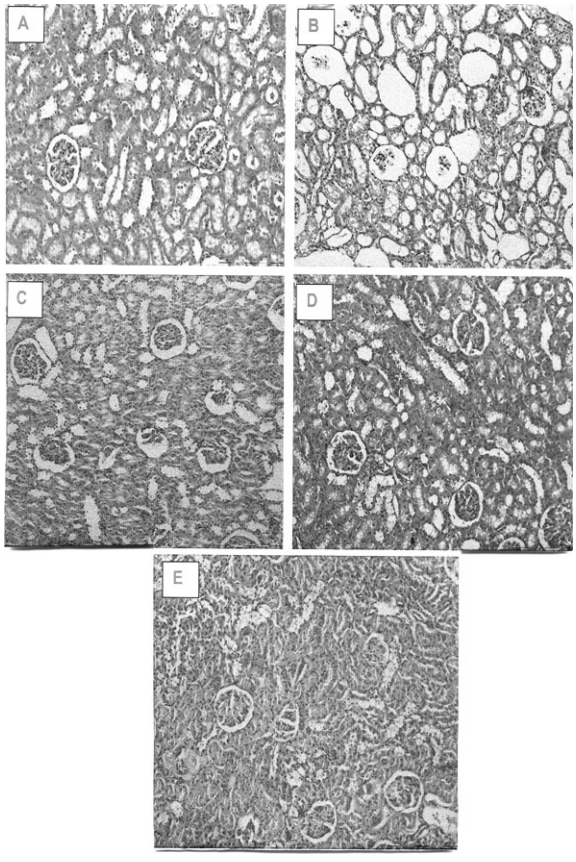

Figure 1: **A** is a section from the kidney of a non-diabetic control rat (group 1) showing normal morphological structures of the renal tubules and glomeruli. **B** is a kidney section of an untreated diabetic control rat (group 2). The histopathological examination revealed various diabetic-associated renal lesions such as necrosis of epithelium, mesangial expansion, and tubular basement membrane thickening. **C** is a kidney section of a diabetic rat that received 0.2 g/kg body weight of honey (group 3). This kidney section shows some improvements compared with Slide **B.** However, it still demonstrates the presence of some morphological changes such as mild mesangial expansion and basement membrane thickening. **D** is a kidney section of a diabetic rat that received 1.2 g/kg body weight of honey (group 4) showing a near-normal morphology of the renal tubules and glomeruli. **E** is a kidney section of a diabetic rat that received 2.4 g/kg body weight of honey (group 5) showing a near normal morphology of the renal tubules and glomeruli.

been reported to stimulate glucokinase, which promotes hepatic glucose uptake and glycogen storage [25]. These two effects will in turn decrease blood glucose. Moreover, honey contains mineral ions such as zinc and copper, which have been shown to exert antihyperglycemic effect in experimentally drug-induced diabetes mellitus. Zinc has been reported to improve insulin sensitivity thereby lowering blood glucose [26], while copper has been shown to decrease blood glucose and levels of lipid peroxidation in diabetic mice [27]. Zinc and copper are also essential minerals required for insulin and glucose metabolism. Therefore, it is possible that fructose, zinc, copper, and other constituents in honey may be involved in mediating the antihyperglycemic effect of honey.

Persistent hyperglycemia is the major hallmark of diabetes. Excessive levels of glucose reaching the mitochondria lead to an overdrive of the electron transport chain. This results in excess formation of superoxide anions normally scavenged by mitochondrial SOD. However, when the latter fails, oxidative stress develops. Superoxide dismutase is undoubtedly important in the regulation of oxidative stress in diabetes mellitus. However, there is variation as to the activity of this enzyme in diabetes mellitus. Some studies have reported reduced SOD activity [28] while others have shown increases [29] or no change in the enzyme [30]. In the present study, there was up-regulation of superoxide dismutase activity in untreated diabetic rats. SOD catalyzes the conversion of superoxide anions to hydrogen peroxide and molecular oxygen. Increased SOD activity in relation to other antioxidant defense mechanisms that remove organic peroxides is detrimental to tissues [31]. Based on our results, it can be implied that in overwhelming oxidative stress, up-regulation of SOD activity is not necessarily protective, but rather a compensatory response in order to scavenge excess superoxide anions [28]. This is further corroborated by the concomitant increase in TBARS levels and significant decrease in TAS in the untreated diabetic rats. Treatment with honey significantly restored the activity of SOD in a dose-dependent manner. We found that the activities of CAT and GPx in the kidney of diabetic rats were significantly reduced. This is consistent with previous reports [28]. The reduced activities of CAT and GPx may not be unconnected with the increased SOD activity. Increased SOD activity will lead to excess generation of hydrogen peroxide, which is usually converted by CAT and GPx. Continuous and increased generation of hydrogen peroxide beyond the levels that CAT and GPx can scavenge will overwhelm these enzymes, resulting in their reduced activities. Furthermore, excess superoxide anions and hydrogen peroxide have been reported to inhibit CAT and GPx [32]. We observed that the honey-treated diabetic rats had significantly increased activities of CAT and GPx. Our data further showed that the activity of glutathione reductase was reduced in diabetic rats. This is consistent with findings of other researchers [33].

Reduced GR activity is an indication of impaired oxidized glutathione (GSSG) reduction to GSH. Tualang honey increased the activity of GR in diabetic rats. This may imply enhanced GSH regeneration in the honey-treated diabetic rats. Our results also showed that activity of glutathione-S-transferase was significantly decreased in diabetic rats. This is similar to previous findings [33]. Glutathione-S-transferase catalyzes the conjugation to GSH of a large variety of electrophilic alkylating compounds such as 4-hydroxy alkenals and other activated alkenes, which are the products of the oxidative damage of biological membranes and macromolecules [34]. Tualang honey significantly increased the activity of GST. Therefore, this indicates that enhanced GST activity in the honey-treated groups may result in efficient metabolism of these harmful aldehydes. This is corroborated by the reduced levels of MDA in the honey-treated groups.

Enhanced lipid peroxidation, as evidenced by increased TBARS levels, is a direct indicator of cell membrane damage in the kidney of diabetic rats. Similar results have been reported in diabetic animal models and patients [35]. The decrease in CAT and GPx activities in the diabetic rats might have resulted in increased hydrogen peroxide generation leading to elevated levels of reactive oxygen species (ROS), which would subsequently cause lipid peroxidation. Tualang honey significantly decreased the elevated levels of TBARS. This shows that the honey-treated diabetic rats had reduced lipid oxidative damage. Total antioxidant status (TAS) is a sum total of interactions among the various antioxidant components. It indicates the capacity to defend against free radical damage more precisely than the measurement of individual antioxidants. The fact that TAS levels were significantly decreased in untreated diabetic rats shows an impairment of the antioxidant defense system in diabetes mellitus [11]. It was observed that treatment with honey caused a significant increase in TAS. This implies that the honey-treated diabetic rats had better antioxidant defense system to protect against oxidative stress.

Increased mesangial matrix, thickening of glomerular basement membrane, and vacuolation of renal tubules are evident in the early stages of diabetes mellitus [36]. Our results revealed morphological changes such as mesangial matrix expansion, thickening of glomerular basement membrane, and other structural changes in the kidneys of untreated diabetic rats. The honey-treated groups showed improved morphological structures such as reduced mesangial matrix expansion and decreased thickening of glomerular basement membrane. This implies that honey has a beneficial effect on the process of glomerular damage. Although further studies are required to elucidate the mechanism by which honey reduces renal damage, strong evidence supports the role of ROS in activating and triggering several major signal transduction pathways involved in diabetic nephropathy.

In conclusion, tualang honey produces a moderate hypoglycemic effect and ameliorates oxidative stress in kidneys of streptozotocin-induced diabetic rats. It can be speculated that honey consumption may be beneficial in the management of diabetes mellitus. However, in view of the fact that honey produced weight gain in experimental diabetes mellitus, its supplementation in human (especially obese) diabetic patients may necessitate a dose adjustment and reduced calorie intake.

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