ANTIOXIDATIVE PROPOLIS FROM *HETEROTRIGONA ITAMA* AMELIORATES THE EARLY MACROVASCULAR CHANGES IN DIABETIC RATS

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Abstract

Background: Oxidative damage culminates in diabetic macrovascular complication. Ethanolic extract propolis of *Heterotrigona itama* exhibits antioxidative, antidiabetic and cardioprotective properties. However, vasoprotective activity has yet to be reported. This research was designed to investigate the effects of the ethanolic extract of propolis on histology and oxidative stress parameters in the aorta of diabetic rats.

Methods: Raw propolis from Kelantan bee farm was ground, dissolved and distilled with ethanol into crude extract. Adult male Sprague Dawley rats were divided into four groups, n=8/group: normoglycemia (NG), untreated diabetes (DM), metformin-treated diabetes (DM+M, 300 mg/kg/day) and propolis-treated diabetes (DM+P, 300 mg/kg/day). A single injection of intraperitoneal streptozotocin (60 mg/kg) was used to induce diabetes mellitus, followed by a four-week treatment, via oral gavage, administered immediately after successful diabetes-induction with evidence of hyperglycaemia. Both NG and DM groups were given one millilitre of the vehicle. Rats were sacrificed at the end of the experiment; blood was collected for determination of serum lipid profile; the aorta was fixed and processed for histomorphology and histomorphometry evaluation; aortic tissue homogenate assay for oxidant/antioxidant activities. Differences in the parameters between groups were analysed using one-way analysis of variance (ANOVA) with post-hoc adjustment.

Results: Propolis treatment elevated the high-density lipoprotein level and suppressed protein oxidation in diabetic rats. Furthermore, propolis halted the thickening of tunica intima and aberrant proliferation of vascular smooth muscle cells in tunica media.

Conclusion: In conclusion, this proof-of-concept study indicated the potential of propolis as a vasoprotective agent against the early pathogenesis of diabetic macroangiopathy through anti-oxidative and anti- hyperglycaemic activities.

Keywords: Stingless Bee Propolis, *Heterotrigona itama*, Antioxidant, Diabetic Macroangiopathy, Antiatherogenic Effect

Introduction

Diabetes mellitus is a chronic disease with persistent hyperglycaemia due to disrupted energy metabolism. An absolute, or relative insulin deficiency, results in diabetic-related vascular, neurological and end-organ failures. Diabetes mellitus affected 422 million people in 2014, and the worldwide prevalence is expected to leap to 642 million in 2040 (1).

Diabetic cardiovascular disease is characterised by an imbalance of redox homeostasis with the overproduction of reactive oxygen species (2). Chronic hyperglycaemia in diabetes mellitus impairs mitochondrial function with

accelerated production of free radicals in blood vessels. Eventually, oxidative stress overwhelms endogenous antioxidative defence leading to inflammatory reactions, vascular function impairment and atherosclerosis (3, 4).

Temporal progression of the atherosclerotic aortic lesion in type 1 diabetes mellitus has been studied in rabbits over a six-month period (5). Chronic hyperglycaemia induced intimal thickening by vascular smooth muscle proliferation or lipid deposition. The inner arch of the aorta, as an atherosclerosis prone area, demonstrated intima thickening by increase in lipoprotein deposition (6).

Oxidation of protein side chains produces carbonyl groups that mark the level of oxidative stress in many pathological conditions, including diabetes mellitus (7). Malondialdehyde (MDA) is accumulated in tissue as the end product of lipid peroxidation (8) and associated with DM complications (9). The level of glutathione-S-transferase elevated in the aortic tissue of diabetic rats is a marker for oxidative stress (10).

Stingless bees are ubiquitous eusocial bees of the tropics. Their propolis is a sticky resinous substance of floral origin that boosts hive defence. Raw propolis requires further extraction before domestic use. The chemical composition of propolis varies depending on the geographical origin and forestation around the beehives. Furthermore, different extraction methods affect the ethnopharmacological properties of propolis. Altogether, these pose a difficulty in the standardisation of propolis formulation (11, 12).

Nonetheless, there are emerging laboratory investigations on the antioxidative (13), antidiabetic, hypolipidaemic (14), cardioprotective (15) and vasoactive (16) activities of the ethanolic extract of *Heterotrigona itama* propolis. These reports suggest that there is potential pharmacological effect of stingless bee propolis against the diabetes-related vascular damage; this leads to the hypothesis of its *in vivo* vasoprotective activity, which has not been previously studied. Therefore, this research aims to investigate the effect of the ethanolic extract of propolis (from *H. itama*) on the histology and oxidative stress parameters in the aorta of streptozotocin-induced diabetic rats.

Materials and Methods

Preparation of the extract

Extraction was performed in accord with the published protocol (17). Raw propolis (from *H. itama*) was obtained from Kelantan, Malaysia (6.090432, 102.291131). Then, it was cleaned and grounded before dissolving it into 70% ethanol (100 mL/30 g). The resultant solution was filtered sequentially, and the filtrate was concentrated in the rotary evaporator at 60°C. The ethanolic extract of propolis was collected, lyophilised and stored at -20°C for use in experiments.

Ethics statement

All experiments were performed in compliance with the guidelines for the welfare of experimental animals by the Care and Use of Animals for Scientific Purposes. The use of adult male Sprague Dawley rats aged 10-12 weeks old was approved by the Universiti Sains Malaysia (USM) Institutional Animal Care and Use Committee (Approval Number: USM/IACUC/2018/ (112) (922)).

Animals

Thirty-two male Sprague Dawley rats, 12 weeks of age, were individually housed in polypropylene cages with stainless steel covers and maintained under controlled room temperature (25 ± 2°C) and humidity (60 ± 5%) with 12/12 h light/darkness conditions throughout the study. Male rats were selected to eliminate variations in food intake due to ovarian hormones and their faster growing rate than females which enable easier detection of body weight changes. All rats were acclimatized for a week with a normal pellet diet (Altromin 1324, Brogaarden, Denmark) and water *ad libitum*.

Chemical induction of diabetes

After acclimatisation, induction of diabetes was performed using streptozotocin (STZ) (Sigma Aldrich, the United States) diluted in the ice-cold normal saline vehicle. All rats were fasted overnight before the procedure. Rats in the diabetic groups (both treatment and control) received a single intraperitoneal (IP) dose of STZ, 60 mg/kg, whereas the rats in the normoglycaemia control group were given an equal volume of vehicle. Post-STZ induction, the animals were given free access to D-glucose solution, 5%, to prevent fatal hypoglycaemia. Fasting blood glucose (FBG) was determined in the blood samples obtained from the tail vein using a glucometer (Right TD-4279, TaiDoc Technology Corporation, Taiwan) after 72 hr. Diabetes was confirmed by the presence of hyperglycaemia (FBG>8.3 mmol/L) (18).

Allocation of research groups

After establishment of a diabetic model, the rats were randomly assigned into four groups, n=8/group as below:

1. Normoglycemia group as the negative control (NG, one millilitre distilled water/day)
2. Untreated diabetes group as the negative control (DM, one millilitre distilled water/day)
3. Metformin-treated diabetes group as positive control (DM+M, 300 mg/kg/day)
4. Propolis-treated diabetes group as the test group (DM+P, 300 mg/kg/day)

Both metformin and propolis were diluted with distilled water before being administered via oral gavage. Four-

week treatments were initiated immediately after successful DM induction. Weekly body weight, food and water consumption and FBG were recorded. Total body weight gain (BWG), final-week food intake and water intake and final FBG were reported.

Blood and tissue collection

At the end of the experiment, the rats were euthanised with a lethal dose of sodium pentobarbital (300 mg/kg, IP). The blood sample was collected via cardiac puncture (23 G needle) into a plain tube to obtain serum for determination of lipid profile. The whole length of the aorta sample was excised along the spine. The arch of the aorta was used for histology study (6); thoracic aorta was processed for determination of oxidative stress parameters.

Determination of lipid profile

Triglyceride (Tg) was determined from the reaction by glycerol 3-phosphate oxidase (GPO) (19). Total cholesterol (Tc) was estimated using the cholesterol oxidase/peroxidase (CHOD/POD) method (20) with additional precipitation by phosphotungstic acid/$MgCl_2$ to detect high-density lipoprotein cholesterol (HDL-c) (21).

Histomorphology and histomorphometry

The arch of the aorta was fixed by immersion into formaldehyde solution, 10% for 48 hr. Then, the tissue specimens were processed, embedded, sliced horizontally, stained and mounted on glass slides. Staining by haematoxylin and eosin (H&E) and Verhoeff-Van Gieson (VVG) were performed on slides. Haematoxylin and eosin staining visualised tissue architecture (22); VVG identified elastin fibre in aortic tissue (23). Specimen slides were anonymised using code to prevent bias during evaluation with the aid of the image analyser (Olympus BX41, Japan).

The thickness of the tunica intima (TI) and tunica media (TM) and the intima-to-media ratio (IMR) were measured; the number of elastic lamellae was counted on H&E-stained slides based on the previous reports (24, 25). The nuclei density of vascular smooth muscle cells (ND) was quantified using grid boxes (2500 μm^2 each); the area of aortic tissue occupying at least 75% of each grid box was counted according to previous reports (26, 27). Tissue histomorphometry was performed at zero-degree, 90°, 180° and 270°C. Averaged values were reported (28). Qualitative description of the interlamellar elastin-to-collagen ratio (EC) was performed on VVG-stained slides (29).

Determination of oxidative stress parameters

Blood and adipose tissue were removed from the thoracic aorta before processing into tissue homogenate, 10%, using ice-cold phosphate buffer saline. Then, tissue samples were spun with 2000 x *g* at 4 °C for 10 min. The supernatants were obtained and aliquoted for further assay. Tissue level of oxidants (malondialdehyde (MDA) and protein carbonyl (PCO)) and antioxidant glutathione-S transferase (GST) were analysed.

The protein concentration in the sample supernatants was determined using Protein Assay Kit (QCPR-500, QuantiChromTM, BioAssay System, the United States) based on the Bradford assay (30). The concentration of MDA was determined using reaction with 2-thiobarbituric acid (TBA) to form a pink-coloured complex that measured spectrophotometrically at 523 nm (31). Tissue level of protein carbonyl content was analysed using Protein Carbonyl Colorimetric Assay Kit (10005020, Cayman Chemical, the United States) based on the reaction with 2,4-dinitrophenylhydrazine (DNPH) to produce a coloured hydrazone complex that measured spectrophotometrically at 370 nm (32). The activity of GST was estimated through the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH) to produce changes in optical density at 340 nm (33).

Sample size calculation

Power analysis for one-way ANOVA with five groups was conducted in G*Power version 3.1, to evaluate a sufficient sample size using an alpha of 0.05, a power of 0.80 and an effect size of 0.60. These are based on Cohen`s guidelines, which state that an effect size of 0.50 is considered a medium effect. For this study, the effect size of 0.60 was chosen for objectives number one until five because we believed that there will be a huge mean difference result in effect among the groups. So, based on the aforementioned assumptions, the desired sample size is 24 (n=6/group). A drop-out of 20% is expected; therefore, the final calculated sample size is 32 (n=8/group).

Data analysis

Qualitative data were computed into Prism 7 for Mac OS X Version 7.0a (GraphPad Software, Inc., the United States) for analysis using one-way analysis of variance (ANOVA) with post-hoc Tukey's multiple comparison tests with adjustment. Data were presented as means (standard deviation). Total BWG, FI, WI, final FBG, Tg, Tc and HDL-c are tabulated in Table 1. Histomorphology of aortic cross-sections is compiled in Figure 1 and 2. Data on TI, TM, IMR, number of elastic lamellae and ND are presented in Figure 3; aortic MDA, PCO and GST of rats are shown in Figure 4.

Results

Physical characteristics, fasting blood glucose and lipid profile

At the end of the four-week experiment, the DM group experienced significant weight loss, higher final food and water intake and FBG, compared to the NG group, as shown in Table 1. Both metformin and propolis treatments reverted these changes significantly compared to the DM group. There was no significant difference in lipid profile between the NG and DM groups. However, the DM+P group had substantially higher Tc and HDL-c levels compared to the DM group.

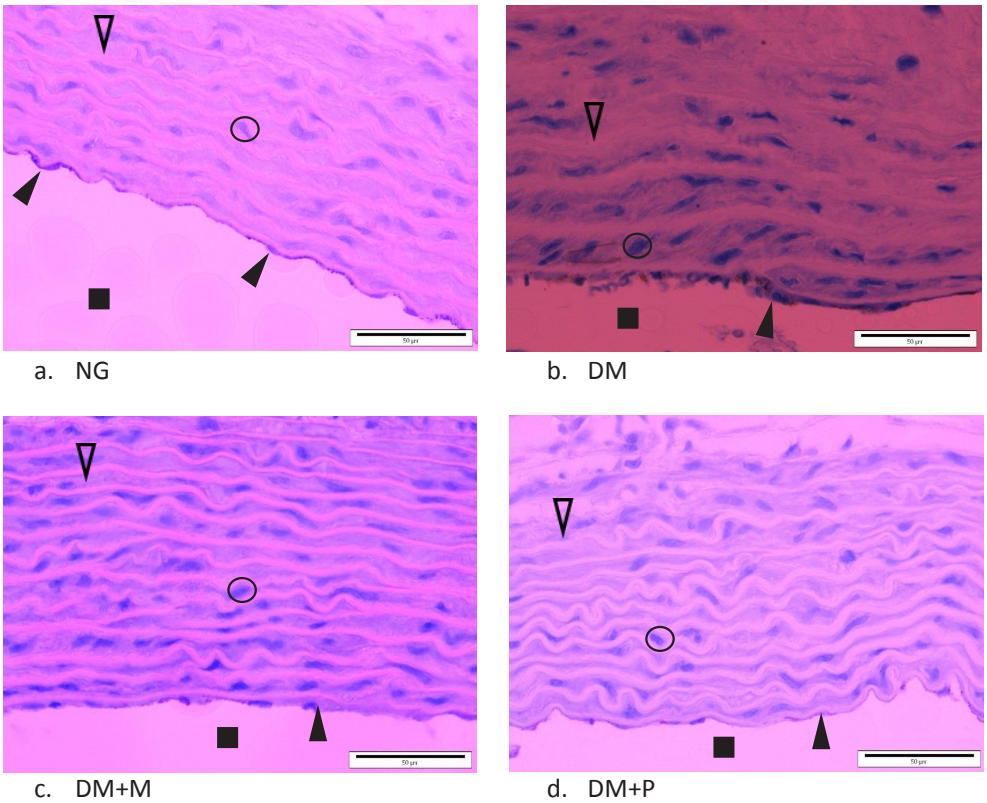
Table 1: Physical characteristics, fasting blood glucose and lipid profile of rats, n=8/group

Parameters	NG	DM	DM+M	DM+P
Body weight gain, g	58±14	65±23[a]	45±29[b]	24±13[a,b]
Final food intake, g	160±9	235±9[a]	193±13[a,b]	197±13[a,b]
Final water intake, mL	283±21	859±50[a]	550±23[a,b]	582±42[a,b]
Final fasting blood glucose, mM	4.1±0.2	26.3±3.4[a]	9.6±1.6[a,b]	11.9±0.4[a,b]
Triglycerides, mM	0.3±0.1	0.4±0.4	0.8±0.4[a]	0.5±0.2
Total cholesterol, mM	1.8±0.3	1.5±0.3	1.6±0.1	1.8±0.2[b]
High-density lipoprotein cholesterol, mM	0.8±0.1	0.7±0.1	0.8±0.1	0.9±0.1[b]

Data were presented as mean (standard deviation). NG, normoglycemia; DM, untreated; DM+M, metformin-treated diabetes; DM+P, propolis-treated diabetes. [a] $p<0.05$ when compared to NG group, [b] $p<0.05$ when compared to DM group

Histomorphology and histomorphometry of aorta

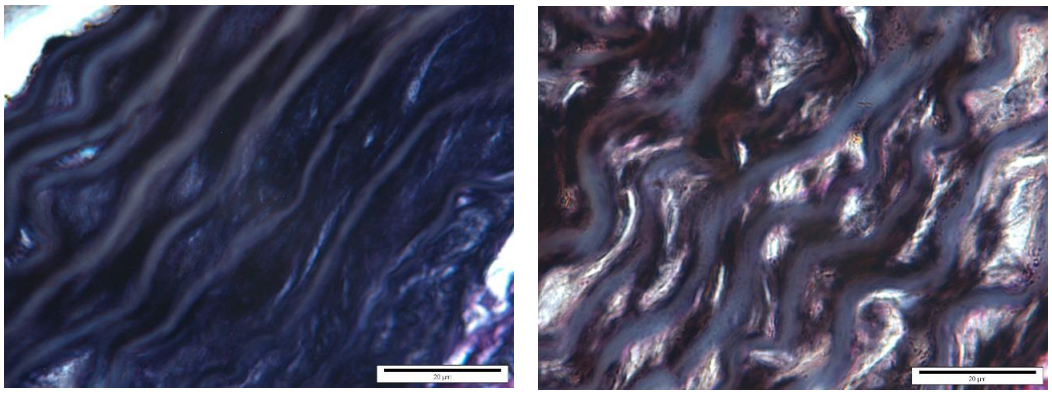
The aortic section from the DM group (Figure 1b) had thicker tunica intima with higher cellularity in tunica media compared to the NG group (Figure 1a). When comparing treatment groups to the DM group, only DM+P group (Figure 1d) exhibited discernible improvement to these pathological changes. The degradation of interlamellar elastin fibres (stained blue to black) was seen in all diabetic groups (Figures 2b–c) with the deposition of collagen (stained pale red to pink) compared to the NG group (Figure 2a). Both treatments alleviated changes observed in the DM group (Figure 2b); however, aortic features of the DM+P group (Figure 2d) resembled that of the NG group. Therefore, the EC in DM+P group was relatively preserved and comparable to the NG group. Histomorphometry data of aorta in Figure 3 supported the qualitative evaluation in Figures 1 and 2. The DM+P group had significantly lower TI and IMR compared to other study groups (Figures 3a–c). There was no difference in aortic TM among animals (Figure 3b); the DM+M group had a significantly higher number of elastic lamellae compared to the NG group (Figure 3d). There was higher ND in the DM group compared to the NG group (Figure 3e). Administration of treatments alleviated these alterations, to a more considerable extent in the DM+P group.



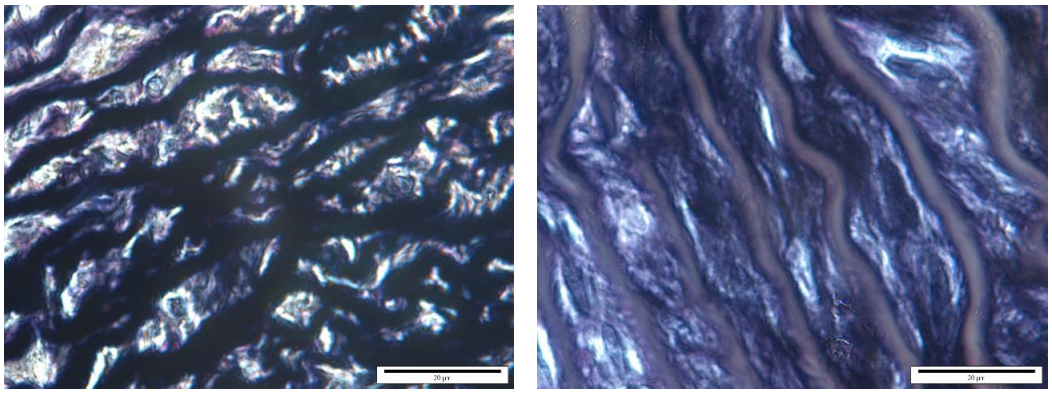
a. NG b. DM

c. DM+M d. DM+P

Figure 1: Histopathological study on sections of rat aorta, n=8/group. Figures 1a to 1d are aortic sections of group NG, DM, DM+M and DM+P, respectively, with H&E staining and viewed under magnification of 400x. NG, normoglycemic control; DM, diabetic control; DM+M, diabetic with metformin treatment; DM+P, diabetic with propolis treatment. Note the thin tunica intima layer and low smooth muscle nucleus density of DM+P compared to DM and DM+M. Solid square ■, aorta lumen; solid arrow head ▼, tunica intima; empty circle ◯, smooth muscle nucleus; empty arrow head ▽, elastic lamella. Scale bar = 50 µm

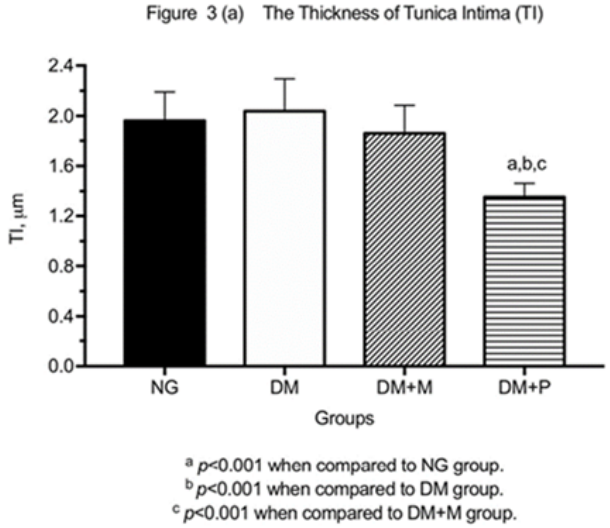


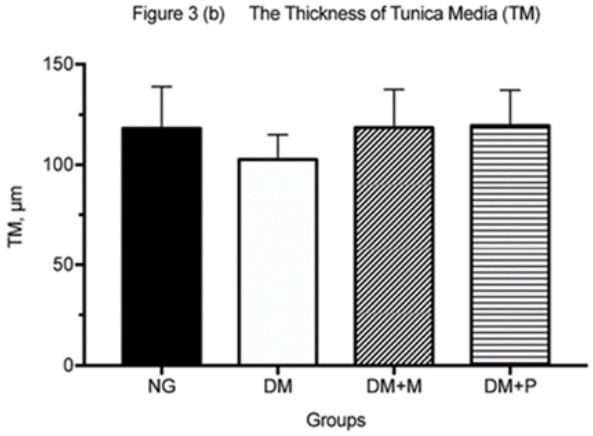
a. NG b. DM

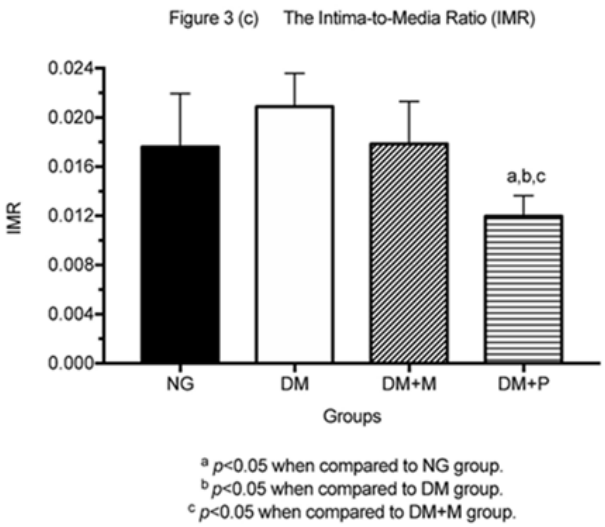


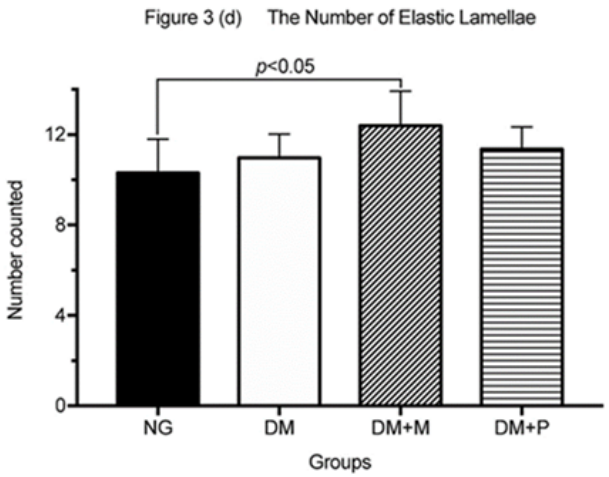
c. DM+M d. DM+P

Figure 2: Histopathological study on sections of rat aorta, n=8/group. Figures 2a to 2d are aortic sections of groups NG, DM, DM+M and DM+P, respectively, with Verhoeff Van Gieson (VVG) staining and viewed under magnification of 1000x. NG, normoglycemic control; DM, diabetic control; DM+M, diabetic with metformin treatment; DM+P, diabetic with propolis treatment. Note the marked decrease in interlamellar elastin fibre (blue to black colour) density and increased collagen (pale red colour) deposition in DM compared to NG. These changes can also be seen, in decreasing severity, in DM+M and DM+P. Scale bar = 20 µm









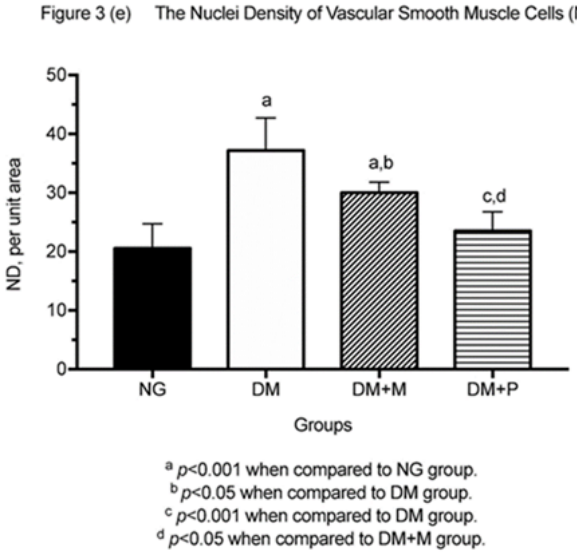


Figure 3: Histomorphometry data of rat aorta, n=8/group. Figure 3 (a) The Thickness of Tunica Intima (TI) 91x75 mm (300 x 300 DPI), Figure 3 (b) The Thickness of Tunica Media (TM) 75x53 mm (300 x 300 DPI), Figure 3 (c) The Intima-to-Media Ratio (IMR) 92x75 mm (300 x 300 DPI), Figure 3 (d) The Number of Elastic Lamellae 78x58 mm (300 x 300 DPI), Figure 3 (e) The Nuclei Density of Vascular Smooth Muscle Cells (ND) 98x89 mm (300 x 300 DPI). NG, normoglycemia; DM, untreated diabetes; DM+M, metformin-treated diabetes; DM+P, propolis-treated diabetes. (a) and (c): Propolis treatment significantly prevented the thickening of tunica intima in diabetic rats. (b): No significant difference in thickness of tunica media among animals. (d): Metformin treatment resulted in significantly higher number of elastic lamellae in aorta. (e): The aorta of diabetic rats exhibited significantly denser vascular smooth muscle cells compared to normal rats. Both treatments significantly reversed this change; the DM+P group showed reduction compared to the DM+M group

Oxidative stress parameters

According to Figure 4a, there was no significant difference in aortic MDA concentration at the end of the experiment. Whereas, the level of PCO and GST were higher in the DM group compared to the NG group. The DM+P group had a significantly lower abundance of PCO and GST compared to the DM group (Figures 4b–c).

Discussion

Physical characteristics, fasting blood glucose and lipid profile

The overt diabetic phenotypes of wasting, polyphagia and polydipsia were observed four weeks after STZ induced diabetes in the DM group. Both insulin deficiency and

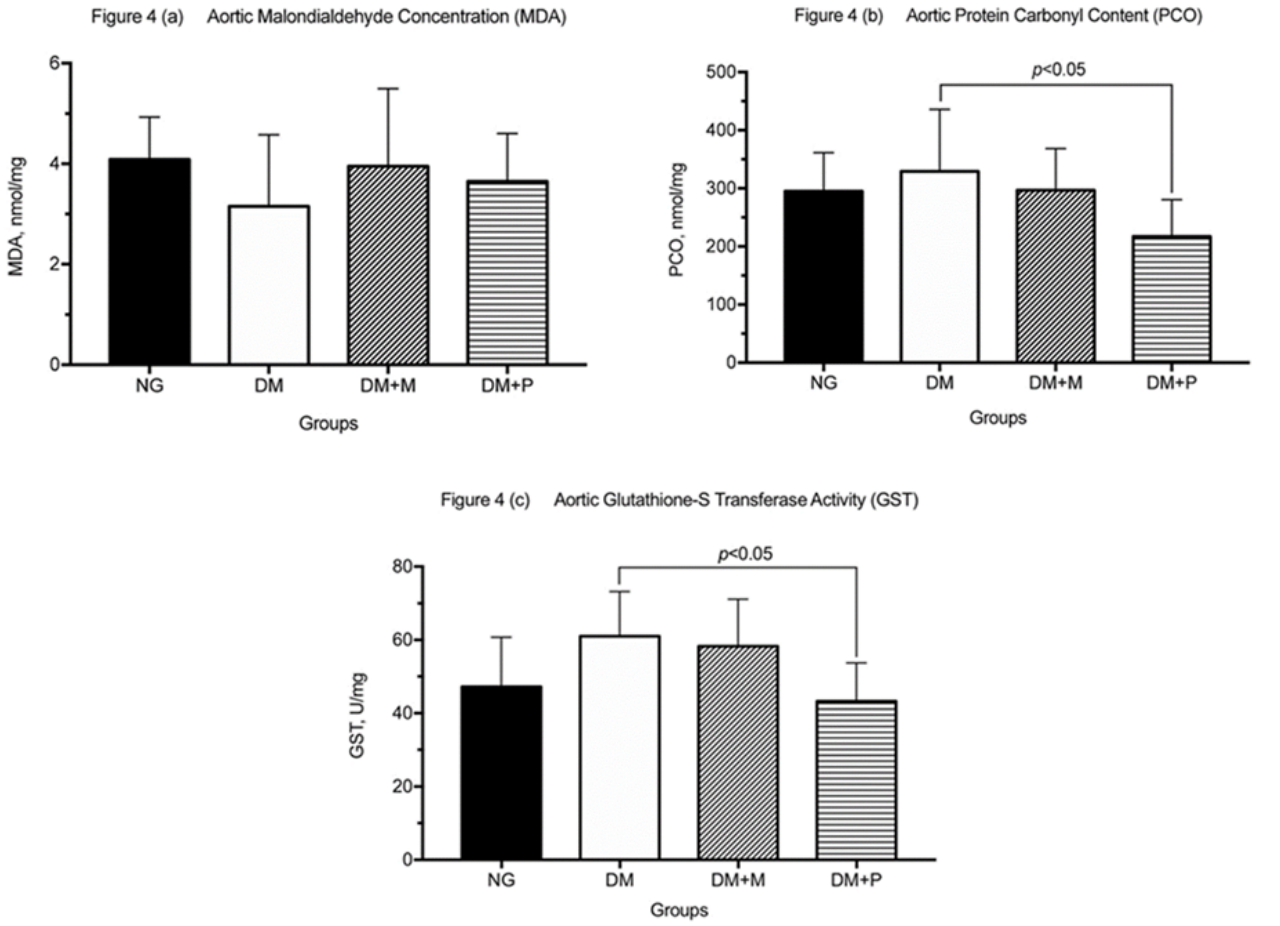


Figure 4: Oxidative stress parameters in the aorta of rats, n=8/group. Figure 4 (a) Aortic Malondialdehyde Concentration (MDA) 75x53 mm (300 x 300 DPI), Figure 4 (b) Aortic Protein Carbonyl Content (PCO) 77x53 mm (300 x 300 DPI), Figure 4 (c) Aortic Glutathione-S Transferase Activity (GST) 78x54 mm (300 x 300 DPI). NG, normoglycemia; DM, untreated diabetes; DM+M, metformin-treated diabetes; DM+P, propolis-treated diabetes. (a): No significant difference in the level of aortic malondialdehyde between groups as a marker of lipid peroxidation. (b): Treatment with propolis prevented the rise in aortic protein carbonyl content seen in diabetes mellitus. (c): Propolis treatment resulted in lower aortic glutathione-S transferase activity compared to diabetic rats.

persistent hyperglycaemia are the pathophysiological basis of these presentations. The impairment of insulin-stimulated glucose uptake in Glucose Transporter 4-rich muscles and adipose tissues leads to catabolic states and disrupted glucose homeostasis (34). A deficiency of insulin in the central nervous system (CNS) causes the upregulation of orexigenic regulators, the neuropeptide Y (NPY), which underlies hyperphagia in diabetes (35). Hyperglycaemia-induced glomerular hyperfiltration coupled with osmotic diuresis lead to dehydration and thirst, and eventually polydipsia (36). Treatment with propolis extract alleviated these abnormalities, which could be explained by the pancreatic preserving effect of phenolic-rich content in propolis in maintaining insulin level in the type 1 diabetic rats (37).

From previous studies, dyslipidaemia is evident after six to eight weeks of STZ-induced diabetes (38-40). A shorter duration (four weeks) of the current study could be the plausible explanation for a lack of statistical difference in lipid profile between the NG and DM groups. The DM+M group recorded a significantly higher Tg level, which contrasted with the outcome of the short-term metformin treatment at a higher dose of 500 mg/kg, which reported a Tg lowering effect (41). However, this finding is difficult to translate into clinical practice because metformin is an off-label drug for type 1 diabetes mellitus and a meta-analysis of existing trials revealed minimal signal between metformin and lipid profile (42).

This study reproduces the findings from both pre-clinical (43) and clinical (44) data that showed that propolis supplementation improves HDL-c level. Furthermore, a previous animal study showed that flavonoid-rich propolis supplementation to healthy rats suppressed total cholesterol and inflammatory markers (45). A recent scholarly review tabulated the major chemical compounds with their corresponding antidiabetic, antioxidative and anti-inflammatory effects; which altogether postulate the inhibitory mechanism of propolis on atherosclerosis (46).

Histomorphology and histomorphometry

The DM+P group showed the preservation of aortic tissue structural integrity. An elevated TI and IMR represent the atherogenic changes in the aorta with infiltration of inflammatory and foam cells which can be ameliorated by anti-inflammatory treatment (47). Previously, chemical fingerprinting of *H. itama* propolis (48-50) revealed flavonoids such as quercetin and kaempferol, which confer antioxidative and anti-inflammatory protective effect on the cardiovascular system (51, 52). Indeed, quercetin selectively inhibited the proliferation of the intimal-type vascular smooth muscle cells *in vitro* as the initial pathogenesis of atherosclerosis.

Aortic TM is related to the body weight in different species of animals. In other words, the number of elastic lamellae, expressed in terms of the lamellar unit is proportionate to the aortic wall stress *in vivo* (53). Vascular remodelling occurs in response to the shear stress in the presence of systemic hypertension (54). The DM+M group, with greater weight gain among the diabetic groups, had a higher number of elastic lamellae implying vascular stiffening. This counterintuitive finding could be inferred from clinical data where metformin use in type 1 diabetes mellitus was associated with higher prevalence of hypertension and obesity (55).

The aorta has higher EC compared to carotid and femoral arteries to maintain higher regional blood flow (56). A low EC links to the reduction in arterial compliance and altered haemodynamic culminating hypertension (57). Similar to a previous report, metformin did not affect arterial compliance (58). Whereas, the DM+P group had a relatively preserved abundance of interlamellar EC, suggesting that propolis maintains the structural integrity of the diabetic aorta.

Furthermore, acute atherogenic insult enhances the mitotic activity and proliferation of vascular smooth muscle cells (59). The enhanced proliferation of aortic vascular smooth muscle cells in the DM group was alleviated by administration of treatments, to a more significant extent, in the DM+P group. The anti-proliferative activity of stingless bee propolis on cancer cells reported previously can be a plausible explanation that warrants further investigation (60).

Oxidative stress parameters

There was a rise in the aortic tissue MDA level in STZ-induced diabetic rats after disease progression for six to eight weeks (61). In this study, aortic MDA level did not show a statistical difference. However, there was an elevation of GST activity in the DM group.

This composite finding leads to the postulation of an early course of diabetic macroangiopathy pathogenesis in compensation. Indeed, GST is a protective antioxidant enzyme that neutralises the negative impact of lipid peroxidation (62); and the aortic level of GST rose in counter-regulating the oxidant insult in STZ-induced diabetes (10). Propolis treatment prevents the reactive rise in GST as a defence against oxidative stress.

The accumulation of tissue protein carbonyl from oxidative damage of aorta in diabetic rats has been reported previously (63, 64). Propolis administration halts *in vivo* oxidative stress-mediated aortic injury through carbonylation of protein. This observation affirms the *in vitro* antioxidative and antidiabetic potential of the propolis of *H. itama* (33).

Conclusion

In conclusion, this pre-clinical proof-of-concept study generates the first reported *in vivo* data on vasoprotection by an ethanolic extract of Malaysian propolis derived from the stingless bee (*Heterotrigona itama*). Propolis administration in STZ-induced diabetic rats yielded an anti-hyperglycaemic effect and modified the early progression of atherogenesis in diabetic macroangiopathy, through modulation of aortic oxidative stress parameters.

Author Contributions

Conceptualization, BSY and NO; data curation, BSY and NO; investigation, BSY; methodology, NO, RZ and ACR; histology supervision, SNHH; original draft preparation, BSY; writing—review and editing, NO, RZ, SNHH, OZL, VUN, MM and ACR. All authors have read and agreed to the published version of the manuscript.

Financial Support

This work was supported by a USM Short Term Research University Grant (304/PPSP/6315123).

Acknowledgment

Authors would like to thank all staff from the Physiology Department (Normawati Ahmad, Noraini Harif and Umeer Rahimi) for the technical support.

Competing Interest

The authors declare no conflict of interest.

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