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Article

Antioxidative Propolis From Stingless Bees (*Heterotrigona Itama*) Preserves Endothelium-Dependent Aortic Relaxation of Diabetic Rats: The Role of Nitric Oxide and Cyclic Guanosine Monophosphate

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Propolis from stingless bees (*Heterotrigona itama*) is a resinous compound that exhibits anti-hyperglycaemia, free radical scavenging, and cardioprotective properties. The effect of propolis on diabetic vessels has not been investigated. Thus, this research aimed to determine the effect of propolis supplementation on the level of antioxidants and its mechanism of action in the aorta of diabetic rats. Male Sprague-Dawley rats were divided into five groups (n=8/group): healthy (control), untreated diabetes (DM), metformin-treated diabetes (DM+M, 300 mg/kg/day metformin), propolis-treated diabetes (DM+P, 300 mg/kg/day propolis extract) and diabetes with combined treatment (DM+M+P, dosage as former). Oral supplementation was conducted for four weeks immediately upon successful induction of diabetes by streptozotocin (60 mg/kg, intraperitoneal injection). At the end of the study, the rats were euthanised, and thoracic aorta was processed into tissue homogenates to determine the levels of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase-1 (GPx-1) and soluble receptor for advanced glycation end-products (sRAGE). Aorta segments were harvested to examine their relaxation response towards graded concentration of acetylcholine (Ach; 10^{-8}–10^{-4}) M following precontraction with phenylephrine (PE; 10^{-6} M). Vasorelaxation towards a cumulative dose of propolis (0.01–1.00%) using PE-precontracted healthy aorta (n=6/experiments) was investigated under various simulated conditions: physiological buffer, L-NAME (10^{-4} M), methylene blue (10^{-5} M), indomethacin (10^{-5} M) and elevated glucose (25 mM). Propolis maintained antioxidative enzymes and sRAGE decoy molecules in the aortic tissue of the diabetic rats. The amelioration of diabetes-induced impairment of endothelium-dependent relaxation by propolis was mediated through the nitric oxide(NO)–cyclic guanosine monophosphate (cGMP) pathway. This non-clinical study reports vasoprotective property of propolis in diabetes mellitus.

Keywords: Propolis. Diabetes Mellitus. Antioxidants. Endothelium-Dependent Relaxation. Nitric Oxide. Cyclic Guanosine Monophosphate.

INTRODUCTION

Diabetes mellitus is an endocrine abnormality that perpetuates metabolic derangement and heightens the risk of atherosclerotic cardiovascular disease (Wannamethee *et al.*, 2011). Chronic hyperglycaemia in diabetes mellitus impairs mitochondrial function through accelerated production of free radicals in blood vessels. Sequentially, vascular oxidative stress overwhelms endogenous antioxidative defence and reduces the activity of endothelial NO synthase (Pitocco *et al.*, 2013). Reduced NO production is linked to the pro-inflammatory and proatherogenic states of vascular dysfunction in diabetes mellitus. Therefore, the search for an agent that hampers this pathophysiological

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cascade will provide a therapeutic opportunity for diabetic vascular disease (Förstermann, 2010).

Propolis is a sticky resin that forms a protective barrier in stingless bees hive. Propolis produced by *H. itama* shows anti-hyperglycaemic (Usman *et al*., 2017), free radical scavenging (Akhir, Bakar, Sanusi, 2017) and cardioprotective (Ahmed *et al*., 2017) properties. These reported bioactivities address the pathogenesis of diabetic angiopathy and lead to the hypothesis that propolis protects against vascular complication and potential multi-target therapeutics in diabetes mellitus. However, there is a lack of data on the effect of *H. itama* propolis on vasculature affected by diabetes mellitus. Therefore, this study aims to determine the level of antioxidants and endothelial-dependent relaxation in the aorta of diabetic rats with propolis supplementation and its mechanism of action.

MATERIAL AND METHODS

Extraction of propolis

Extraction was performed in accord with the published protocol (Usman *et al*., 2017). Raw propolis (*H. itama*) was obtained from Kelantan, Malaysia (6.090432, 102.291131). Then, it was cleaned and grounded before being dissolved into 70% ethanol (100 mL/30 g). The resultant solution was filtered sequentially, and the filtrate was concentrated in a rotatory evaporator at 60 ºC. The ethanolic extract of propolis was collected, lyophilised and stored at –20 ºC for experimental use.

Research animals and induction of diabetes

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]). The experiment was conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Intraperitoneal streptozotocin (60 mg/kg) was used to induce diabetes, and 1mL normal saline (vehicle) was injected to the rats in the non-diabetic groups. The animals were assessed after 72 hours post-induction for the attainment of diabetes mellitus (FBG > 11.1 mM).

Experimental design

The animals were divided into five groups (n=8/group): healthy (control), untreated diabetes (DM), metformin-treated diabetes (DM+M, 300 mg/kg/day metformin), propolis-treated diabetes (DM+P, 300 mg/kg/day propolis extract) and diabetes with combined treatment (DM+M+P, dosage as former). Treatment was administered via oral gavage for four weeks immediately after successful induction of diabetes. All groups received the same volume of 1mL per gavage. The rats were euthanised at the end of the experiment using sodium pentobarbital (200 mg/kg), and blood was collected to determine fasting blood glucose (FBG). Thoracic aorta was dissected and processed into 10% tissue homogenates using ice-cold phosphate buffer saline. Segments of the aorta (2–4 mm) were reserved for tissue bath assay.

Determination of the level of antioxidants in aorta

Aortic tissue homogenates were centrifuged at 10,000 × *g* for 10 minutes, and the supernatant was aliquoted for further assay on protein concentration, SOD, CAT, GPx-1 and sRAGE. Tissue SOD activity was determined on the basis of reaction with riboflavin/nitrotetrazolium blue upon photoactivation (Cheng *et al*., 2015). A protein assay kit (QCPR-500, QuantiChromTM, BioAssay System), Catalase Assay Kit (E-BC-K30, Elabscience®, the United States), Rat GPx-1 ELISA Kit (E-EL-R2491, Elabscience®, the United States) and Rat Advanced Glycosylation End Product Specific Receptor ELISA Kit (E-EL-R0643, Elabscience®, the United States) were used to determine the tissue level of CAT (Hadwan, Abed, 2016), GPx-1 (Luo *et al*., 2015) and s-RAGE (Greco *et al*., 2014). The SOD/(CAT+GPx-1) ratio was calculated as previously reported (Hadzi-Petrushev *et al*., 2018).

Tissue bath assay set-up

An automated organ bath (PanLab, LE01026, the United States), force transducer (ADInstrument, MLT050/D, New Zealand), PowerLab 8/30 (ADInstrument, ML870, New Zealand) and LabChart® Reader software were assembled for tissue bath assay. Then, the set-up was calibrated using a 1 g weight. Krebs-Ringer bicarbonate solution (NaCl, 118.6 mM; KCl, 4.8 mM; $CaCl_2$, 2.5 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 0.2 mM; $NaHCO_3$, 25.1 nM; glucose, 11.0 mM) was utilised as the physiological buffer.

At the beginning of the assay, the aorta was mounted and equilibrated in the tissue bath chamber filled with buffer for 60 mins at a resting tension of 1 g. The buffer

was replaced every 20 mins. Throughout the experiment, the tissue bath chamber was equilibrated at 37 ºC with continuous carbogen (95% oxygen, 5% carbon dioxide) aeration. The viability of aorta was confirmed by the consistent tension increment following two rounds of stimulation with KCL (60 mM) before the experiment (Ulu *et al*., 2010).

Determination of Endothelium-Dependent Relaxation

The cumulative tension reductions towards graded concentration of Ach (10^{-8}–10^{-4} M) in aorta segments precontracted with PE (10^{-6} M) were recorded (Hassan *et al*., 2011).

Determination of the Mechanism of Action of Propolis

Thoracic aorta of healthy rats was dissected and divided into segments. The integrity of the endothelial layer in PE-precontracted viable aorta segments was confirmed by the presence of a 60–80% reduction in tension towards Ach (10^{-6} M) (Ulu *et al*., 2010). Only viable aortic tissues with intact endothelium were used for further assay. Then, the aortic segments (n=6/experiment) were incubated for 30 minutes with enzyme inhibitors before precontraction with PE (10^{-6} M): L-NAME, 10^{-4} M; methylene blue, 10^{-5} M; indomethacin, 10^{-5} M; elevated glucose, 25 mM (Fatehi-Hassanabad *et al*., 2005). Subsequently, relaxation responses towards graded concentration of propolis extract (0.01–1.00%) under each conditions were recorded.

Statistical analysis

Statistical Package for the Social Sciences version 24 and GraphPad Prism version 7.0a were used for statistical analysis. Numerical data were presented as median (interquartile range). Differences between groups were analysed using the Kruskal–Wallis H test with post-hoc Bonferroni correction. The percentage of tension increment from resting baseline and that of tension decrement from plateau were derived from vascular tension tracing. Non-linear regression was then performed to produce dose-response curves, from which the potency, pEC_{50} and maximal tension reduction, E_{max} were derived. The pEC_{50} and E_{max} differences of the propolis extract in the presence of inhibitors compared with the physiological buffer were analysed using the Wilcoxon signed ranks test.

RESULTS AND DISCUSSION

Anti-hyperglycaemic activity of propolis

At the end of this study, the DM group had significantly higher FBG compared with the control group (27.0 [5.8] *vs* 4.1 [0.3] mM). Meanwhile, the DM+M, DM+P and DM+M+P groups showed glycaemic improvement compared with the DM group, with FBG values of 8.9 (2.7), 11.9 (0.5) and 5.6 (0.8) mM, respectively. Chronic elevation of blood glucose, as the disease hallmark of diabetes mellitus, is the key trigger of oxidative stress generation and end-organ damage (King, Loeken, 2004). The current findings reinforce the anti-hyperglycaemic effect of propolis in diabetic rats (Usman *et al*., 2017) and invites further study to elucidate its glucose-lowering mechanism.

H. itama propolis can inhibit alpha-glucosidase, an intestinal enzyme that facilitates carbohydrate hydrolysis and glucose absorption in the gastrointestinal tract (Ibrahim *et al*., 2016). Acarbose is the marketed alpha-glucosidase inhibitor used to manage diabetes mellitus (DiNicolantonio, Bhutani, O'Keefe, 2015). However, the alpha-glucosidase inhibition of *H. itama* propolis has higher potency compared with that of acarbose (Ibrahim *et al*., 2016).

Nevertheless, metformin was chosen as the positive control in this study for two reasons: (1) Metformin reduces aortic oxidative stress and improves ACh-induced aortic relaxation through the involvement of NO in rats with streptozotocin-induced diabetes (Majithiya, Balaraman, 2006). (2) Metformin provides cardiovascular benefits for patients with type 1 diabetes mellitus (Petrie *et al*., 2017).

Level of antioxidants in aorta

Figure 1A–D shows the level of aortic antioxidants in the experimental rats. The aortic SOD (Figure 1A) and CAT (Figure 1B) activities increased significantly in the DM group compared with those in control group. Superoxide dismutase detoxifies intracellular free radicals into hydrogen peroxide, which is then degraded by CAT into water and oxygen. Both antioxidative enzyme activities increase as a compensatory mechanism to protect against heightened oxidative stress in the aorta of diabetic rats (Kakkar *et al*., 1996). The DM+P group had significantly lower SOD activity compared with the DM group, whereas the CAT activity was higher in the DM+M+P group than in the control. Propolis prevents the reactive elevation

of SOD activity in the aorta. Meanwhile, metformin treatment increases the aortic CAT level in diabetic rats (Chukwunonso Obi *et al.*, 2016), and this beneficial effect was augmented by the addition of propolis in this study.

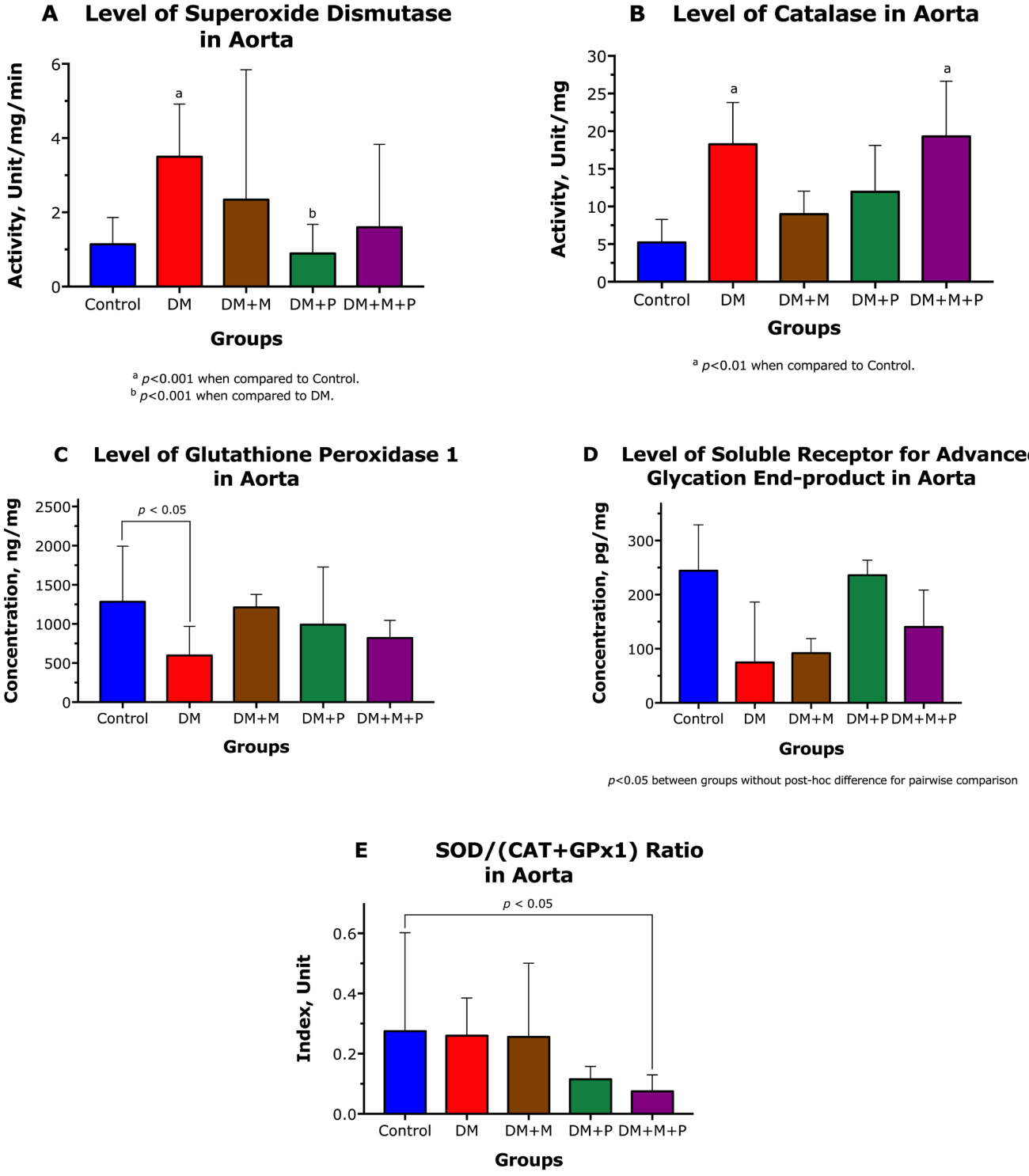
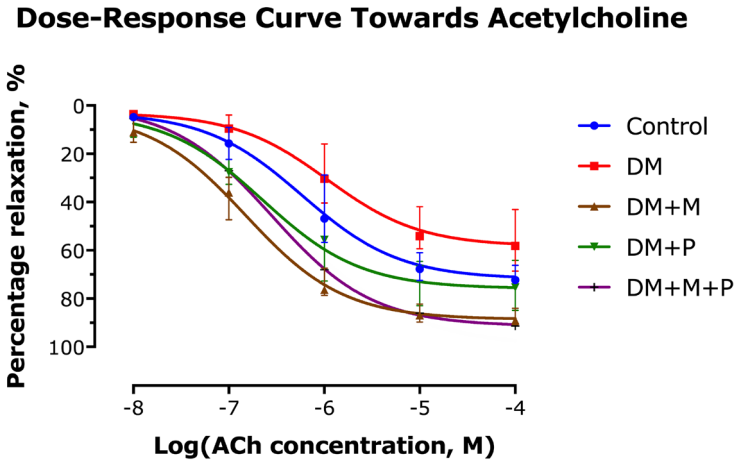


FIGURE 1 – The level of antioxidants in the aorta of experimental rats (n=8/group) comprising: A superoxide dismutase, B catalase, C glutathione peroxidase 1, D SOD/(CAT/GPx1) ratio, E soluble receptor for advanced glycation end-product. Data were presented as median (interquartile range) and analysed using Kruskal–Wallis H test with post hoc Bonferroni correction. CAT, catalase; DM, diabetes mellitus; GPx1, glutathione peroxidase 1; M, metformin; P, propolis; SOD, superoxide dismutase.

The concentrations of GPx-1 (Figure 1C) and sRAGE (Figure 1D) in the aortic tissue were lower in the DM group than in the control group. All treatment groups showed the opposite changes. Glutathione peroxidase scavenges hydrogen peroxide into stable alcohol in the presence of reduced glutathione. Aortic tissue in the diabetic rats show a reduction in both glutathione peroxidase and reduced glutathione (Pari, Monisha, Jalaludeen, 2012). Meanwhile, sRAGE is a decoy receptor that blocks the downstream AGE–RAGE signalling-mediated pro-inflammatory, pro-oxidative and atherogenic vasculopathy. Exogenous administration of sRAGE attenuates chronic vascular inflammation and atherosclerosis in diabetic mice (Wendt *et al.*, 2006), and propolis and metformin restore the levels of GPx-1 and sRAGE in the aorta as defence against oxidative stress. The SOD/(CAT+GPx-1) ratio represents the capability of the first line antioxidative enzymes to remove hydrogen peroxide, which is a source of cellular damage (Hadzi-Petrushev *et al.*, 2018). Both DM+P and DM+M+P groups had low SOD/(CAT+GPx-1) ratios (Figure 1E), representing an *in vivo* antioxidative potential of propolis in preventing the accumulation of hydrogen peroxide.

Endothelium-dependent relaxation of aorta

Figure 2 depicts the dose–response curves towards ACh and the corresponding pEC_{50} and E_{max} in the aorta of the rats. The DM group had reduced relaxation response towards ACh compared with the control group. Diabetic animals have impaired ACh-mediated endothelial-dependent relaxation (Oyama *et al.*, 1986). A recent study demonstrated the duration-dependent deterioration of endothelial function in diabetic rats (Hassan *et al.*, 2011). Endothelial dysfunction in streptozotocin-induced diabetes mellitus affects both small and large vessels (Leo, Hart, Woodman, 2011; Ali, Woodman, 2019). In the present study, all treatment arms exhibited a significant improvement in ACh-induced relaxation compared with that in the DM group. Metformin mediates the restoration of endothelial function in rats with streptozotocin-induced diabetes via NO signalling involving potassium channels (Majithiya, Balaraman, 2006).



	pEC_{50}(ACh)	E_{max}(ACh)
Control	5.5 (0.8)	72 (8)
DM	4.8 (1.3)	58 (25)
DM+M	6.6 (0.5)[a,b]	89 (5)[b]
DM+P	5.7 (0.9)[b]	76 (18)
DM+M+P	6.3 (1.3)[b]	91 (40)[b]

[a] $p<0.05$ when compared to Control.
[b] $p<0.05$ when compared to DM.

FIGURE 2 – Dose–response curve towards acetylcholine (10^{-8}–10^{-4} M) as the indicator of the degree of endothelium-dependent relaxation among experimental rats in tissue bath assay (n=8/group). Non-linear regression of aortic tension to plot four-parameter variable sloops. All data were presented as median (interquartile range) and analysed using Kruskal–Wallis H test with post hoc Bonferroni correction. ACh, acetylcholine; DM, diabetes mellitus; E_{max}, maximal relaxation; M, metformin; P, propolis; pEC_{50}, potency.

Notably, there was no significant difference in the ACh potency of aortic tissues between the DM+P and DM+M groups. Thus, propolis supplementation preserved endothelial function in the diabetic rats. The DM+M and DM+M+P groups achieved statistically higher percentages of relaxation compared with the DM group. The pleiotropic effects of metformin on the improvement of systemic oxidative stress and NO bioavailability, besides glycaemic control, may support this finding. Additionally, there is no significant difference in maximal relaxation response towards ACh between the control and DM+P groups. Therefore, *H. itama* propolis normalised vascular function.

Mechanism of action of propolis-induced aortic relaxation

The pEC_{50} and E_{max} of the propolis extract were 2.88 (0.79)% and 80 (28) %, respectively, as shown in Figure 3A. This study is the first to produce the pharmacodynamic data of dose-dependent vasorelaxation by propolis extract (*H. itama*) in rat aorta. Propolis extract of the Australian stingless bee, *Tetragonula carbonaria* has been found to mediate endothelium-independent vasorelaxation in coronary arteries (Massaro *et al*., 2013). Nonetheless, research and development on propolis represent a niche in modern pharmaceutics. Further research warrants the delineation of the cardiovascular effect of stingless bee propolis from different regions and species with a focus on its vasoactive property.

Figure 3B-E displays the dose–response curves towards propolis extract with their pEC_{50} and E_{max} in healthy aorta segments under various conditions. The pEC_{50} (propolis) was significantly reduced after incubation with L-NAME (Figure 3B), methylene blue (Figure 3C) and elevated glucose. The reduced pEC_{50} (propolis) after exposure to inhibitors of NO synthase and guanylyl cyclase indicated the involvement of the NO–cGMP pathway in propolis-mediated relaxation. The lack of a significant reduction in pEC_{50} (propolis) with indomethacin (Figure 3D), which is an inhibitor of cyclooxygenase enzyme, suggested that the activity of propolis was independent of prostaglandin signalling (Fatehi-Hassanabad *et al*., 2005). Short-term exposure to hyperglycaemia raised the aortic tissue level of hydroxyl radicals, as evidence of oxidative stress, and concomitantly decreased the level of NO and NO synthase activity (Qian *et al*., 2010). Particularly, acute hyperglycaemia (Figure 3E) reduced the potency of propolis, but not the maximal vasodilatory response.

In sum, propolis of *H. itama* possesses anti-hyperglycaemic, antioxidative effects and maintains the level of AGE-scavenging sRAGE in the aorta of rats with streptozotocin-induced diabetes. Functionally, propolis alleviates the impairment of endothelium-dependent relaxation in diabetic rats via the endothelium-dependent NO–cGMP pathway. This non-clinical animal study demonstrated the vasoprotective property of propolis in streptozotocin-induced diabetes mellitus.

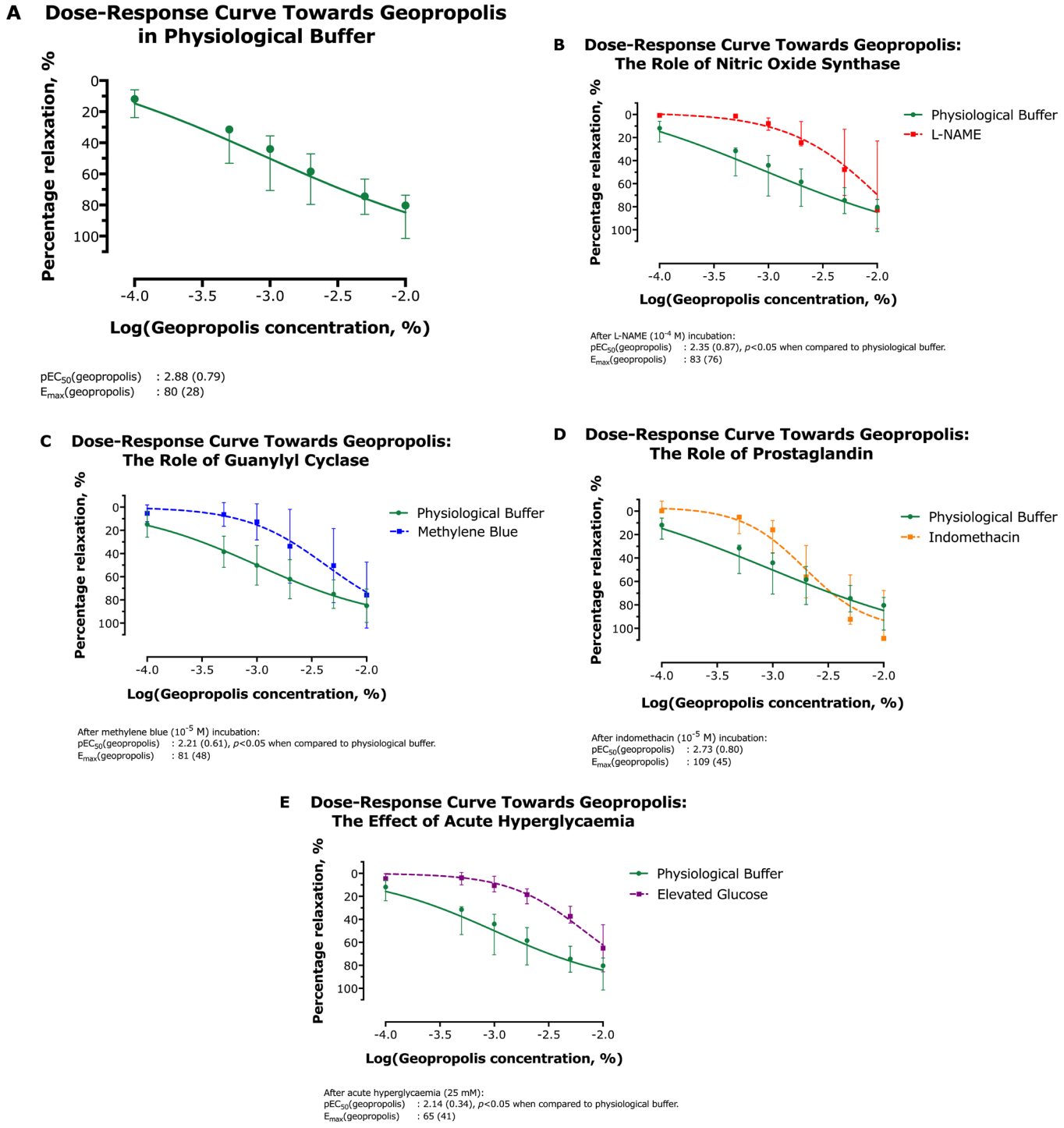


FIGURE 3 – Dose–response curve towards propolis extract under various conditions in the phenylephrine-precontracted aorta (n=6/experiment): **A** physiological buffer as control, **B** L-NAME (10^{-4} M) as nitric oxide synthase inhibitor, **C** methylene blue (10^{-5} M) as guanylyl cyclase inhibitor, **D** indomethacin (10^{-5} M) as cyclooxygenase inhibitor and **E** elevated glucose. Data presented as median (interquartile range) and analysed using Wilcoxon signed ranks test to compare with the control. E_{max}, maximal relaxation; L-NAME, Nomega-Nitro-L-arginine methyl ester; pEC_{50}, potency.

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