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Hibiscus sabdariffa (ROSELLE) POLYPHENOL-RICH EXTRACT PREVENTS THE AORTIC OXIDATIVE DAMAGE IN TYPE 1 DIABETIC RATS

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Graphical abstract

Abstract

Diabetes mellitus complications mainly occur due to the oxidative stress-related condition. Thus, this study aimed to investigate the protective effect of the polyphenol-rich extract of *Hibiscus sabdariffa* (roselle) (HPE) on the aorta of streptozotocin-induced diabetic rats. Twenty-four male Sprague-Dawley rats were divided into four groups (n=6/group); non-diabetes control (NDM), diabetes (DM), diabetes treated with HPE (DM+HPE) and metformin (DM+MET). HPE and metformin were given via oral force-feeding at the dose of 100mg/kg and 150mg/kg respectively for eight weeks duration. At the end of the experiment period, blood pressure was measured before the rats were sacrificed. Blood was taken for experimental of plasma glucose and lipid levels whilst aorta was excised for oxidative stress and histological evaluation. Results showed that HPE treatment reduced plasma glucose level, blood pressure and improved dyslipidemia significantly ($p<0.05$) in DM+HPE group compared to DM group. Malondialdehyde (MDA) and advanced oxidation protein product (AOPP) levels were significantly decreased ($p<0.05$) while reduced glutathione (GSH) level was significantly increased ($p<0.05$) in DM+HPE group compared to DM group. No histological changes observed in the aorta of all diabetic groups compared to NDM group. In conclusion, HPE might have a potential in minimizing of vascular damage in diabetic conditions.

Keywords: Diabetes mellitus, aorta, dyslipidemia, roselle, oxidative stress

Abstrak

Komplikasi diabetes melitus berlaku terutamanya akibat keadaan berkaitan tekanan oksidatif. Oleh itu, kajian ini bertujuan untuk mengkaji kesan perlindungan ekstrak *Hibiscus sabdariffa* (rosel) kaya polifenol (HPE) terhadap aorta tikus diabetes aruhan streptozotosin. Sejumlah 24 ekor tikus jantan Sprague-Dawley dibahagikan kepada empat kumpulan (n=6/kumpulan): kawalan bukan diabetes (NDM), diabetes (DM), diabetes dengan rawatan HPE (DM+HPE) dan diabetes dengan rawatan metformin (DM+MET). HPE dan metformin diberikan secara paksaan oral pada dos 100 mg/kg dan 150 mg/kg untuk tempoh lapan minggu. Pada akhir tempoh kajian, tekanan darah diukur sebelum tikus dikorbankan. Darah diambil untuk pengukuran aras glukosa plasma dan lipid manakala tisu aorta diambil bagi penilaian *oxidative stress* dan histologi. Hasil kajian menunjukkan bahawa rawatan HPE mengurangkan aras glukosa plasma, tekanan darah serta memperbaiki keadaan dislipidemia secara signifikan ($p<0.05$) pada kumpulan DM+HPE berbanding dengan kumpulan DM. Aras malondialdehid (MDA) dan pengoksidaan produk protein lanjutan (AOPP) menurun secara signifikan ($p<0.05$) manakala

aras glutation terturun (GSH) meningkat secara signifikan ($p<0.05$) pada kumpulan DM+HPE berbanding dengan kumpulan DM. Pemerhatian histologi menunjukkan tiada sebarang perubahan struktur tisu aorta pada semua kumpulan tikus diabetes berbanding kumpulan NDM. Kesimpulannya, HPE mempunyai potensi mengurangkan kerosakan vaskular dalam keadaan diabetes.

Keywords: Diabetes melitus, aorta, dislipidemia, rosel, tekanan oksidatif

1.0 INTRODUCTION

Diabetes mellitus is a chronic hyperglycemic condition caused by either the failure of pancreas to produce insulin or reduced insulin sensitivity or both [1]. A study reported that 415 million of adults have diabetes and it is expected to climb up to 642 million in the year 2040 [2]. It is currently known that diabetes is a major cause of morbidity and mortality. Chronic and poorly controlled hyperglycemia would consequently lead to diabetic complications either microvascular such as retinopathy, nephropathy, and neuropathy or macrovascular such as atherosclerosis and cardiovascular diseases (CVD) [3].

CVD is the most common cause of death among diabetic patients. Apart from hyperglycemia, diabetic patients also contracted other comorbidities such as hypertension and hyperlipidemia, thus raising the risk of CVD. The possible mechanisms are through the endothelial dysfunction, excessive reactive oxygen species (ROS) production and high oxidative stress condition [4].

Oxidative stress results from an imbalance between the generation of ROS and defense mechanism activities of antioxidant. The consequence of enhanced ROS production or attenuated ROS scavenging capacity by antioxidants resulted in oxidative tissue damage [5]. In hyperglycemic condition, excess generation of ROS such as superoxide, hydroxyl radicals, and hydrogen peroxides are the result from increased glucose auto-oxidation, polyol, hexosamine and protein kinase C pathways, as well as a production of advanced glycation end products (AGE). Thus, hyperglycemia caused oxidative stress and consequently results in the establishment of diabetic complications including vascular damage [6].

Hibiscus sabdariffa or known as roselle is widely used in the production of food and beverages [7]. Roselle has been shown to possess antihyperglycemic and antihyperlipidemic properties, thus have a potential in preventing CVD [8, 9]. Roselle extract has high polyphenol bioactive compound such as quercetin, hibiscus acid and protocatechuic acid that contribute to its antioxidant properties [10, 11, 12]. Therefore, this study was undertaken to investigate whether the *Hibiscus sabdariffa* polyphenol–rich extract (HPE) is able to prevent the aortic oxidative damage in streptozotocin-induced Type 1 diabetic rats.

2.0 METHODOLOGY

2.1 Preparation of *Hibiscus sabdariffa* Linn. (Roselle) Polyphenol-rich Extract (HPE)

Dried roselle calyces (specimen voucher: UKMB40308) were collected from a local plantation, Ai Agro Marketing, Terengganu, Malaysia. HPE was extracted as described by Peng *et al.* [13]. In brief, five grams of dried calyx powder was boiled in 50 mL of HPLC grade methanol and stirred for 30 min in 60 °C water bath. The extract was then filtered and the same procedure was repeated twice using the filtered residue. The extracts were pooled together and evaporated to dryness. The extract was then added into 10 mL of deionized water (adjusted to pH 2.3 with 0.1 N HCl) and partitioned successively with n-hexane (3 x 10 mL) and ethyl acetate (3 x 10 mL). The ethyl acetate soluble fraction was evaporated to dryness using a rotary evaporator (Buchi, Switzerland). The HPE was stored at -20°C for later use.

2.2 Animals

Twenty-four adult male Sprague-Dawley rats (300-350 g) were supplied by the Animal Unit, Universiti Kebangsaan Malaysia. The animals were kept in metabolic cages at the animal research laboratory, under 12 h light/dark cycles at room temperature condition. All rats were given free access to a standard pellet diet and drinking water. The animal usage had been approved by Universiti Kebangsaan Malaysia Ethics Committee (UKMAEC) (UKMAEC NO: FSK2015/2014/BALKIS/11-FEB./643-FEB.-2015-FEB-2018) and the guidelines were strictly followed.

2.3 Diabetes Induction and Treatment

All rats were fasted overnight before the diabetes induction. Type 1 diabetes mellitus was induced via a single intraperitoneal injection of freshly prepared streptozotocin (STZ) (Sigma Chemicals, ST. Louis, Missouri, USA) at the dose of 55 mg/kg body weight [14]. After three days, rats with the glucose level above 15 mmol/L were selected for the study [15]. The diabetic rats were randomly divided into three groups as followed: diabetic group (DM), diabetic group treated with HPE (DM+HPE) and diabetic group treated with metformin (DM+MET). The non-diabetic rats (NDM)

acted as control group and all groups have an equal number of rats (n=6). HPE and metformin were given at the dose of (100mg/kg) [13] and (150mg/kg) [16] respectively and the treatment was commenced on the same day of diabetic confirmation. HPE and metformin were given via oral force-feeding, daily for eight consecutive weeks. The body weight, food and water intake of each rat were measured weekly. At the end of the experimental period, rats were fasted overnight and blood pressure was measured by using non-invasive tail-cuff measurement (CODA system, Kent Scientific, Conn., USA) before the rats were sacrificed. Blood was taken via orbital sinus for measurement of plasma glucose and lipid levels. The aorta was isolated, cleaned using phosphate buffer saline (pH 7.4) and the surrounding connective tissue was stripped off. Part of the aorta was fixed in 10% formalin for histological study and the remaining was stored at -20°C for oxidative stress evaluation.

2.4 Aorta Homogenate Preparation

The thawed aorta was blotted dry with filter paper and weighed. The aorta homogenates were prepared based on the method described by Upston *et al.* [17].

2.5 Plasma Biochemical Analysis

Plasma glucose level was determined by using the assay kit supplied by Pointe Scientific, Inc. (USA) which was based on the principle of glucose oxidase method. Concentrations of triglyceride, total cholesterol and HDL were measured using semi-automated biochemical analyzer Biosystem BTS 350 (BioSystem Reagent & Instrument, USA).

2.6 Oxidative Stress Evaluation

Oxidative stress evaluation was done using spectrophotometer. Malondialdehyde (MDA) was quantified based on the reaction of MDA with thiobarbituric acid at 100°C to form thiobarbituric acid reactive substances (TBARS) which was measured at 532 nm [18]. Advanced oxidation protein product (AOPP) was measured based on the previous study by Witko-Sarsat *et al.* [19] and measured at 340 nm. Superoxide dismutase (SOD) activity was evaluated based on its capacity to inhibit the reduction of ferricytochrome and the reaction was measured at 560 nm [20]. Reduced glutathion (GSH) was measured at 412 nm following the reaction of homogenate GSH with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) to produce yellow-colored complex [21]. Catalase (CAT) activity was measured based on Aebi's method [22].

2.7 Histological Evaluation

The fixed aorta was dehydrated with the increasing concentration of ethanol, embedded in paraffin to form paraffin blocks. The paraffin blocks were then sectioned at 3 µm thickness and stained with Hematoxylin and Eosin for histological evaluation.

2.8 Statistical Analysis

The data analysis was performed using the *Statistical Packages for the Social Sciences* (SPSS) version 21.0 and expressed as means ± SEM. The significant differences among groups were evaluated using one-way analysis of variance (ANOVA) followed by Post Hoc Tukey's test. Data were considered as statistically significant at $p<0.05$.

3.0 RESULTS AND DISCUSSION

3.1 Effect of HPE Treatment on Body Weight, Food and Water Intake

Body weight, water, and food intake were shown in Figure 1a, Figure 1b and Figure 1c respectively. At the end of eight weeks all diabetic groups exhibited a significant increase in fluid and food intake as well as decrease in body weight when compared to NDM group. The increment of food intake is due to the inability of cells to use glucose as a source of energy [23]. The reduction of body weight is a result of proteolysis and lipolysis due to insulin deficiency [24, 25]. Meanwhile, the hyperglycemic condition caused polyuria and consequently leads to polydipsia. All these manifestations are the consequences of failure in glucose utilization as well as glucose accumulation in blood and urine [26].

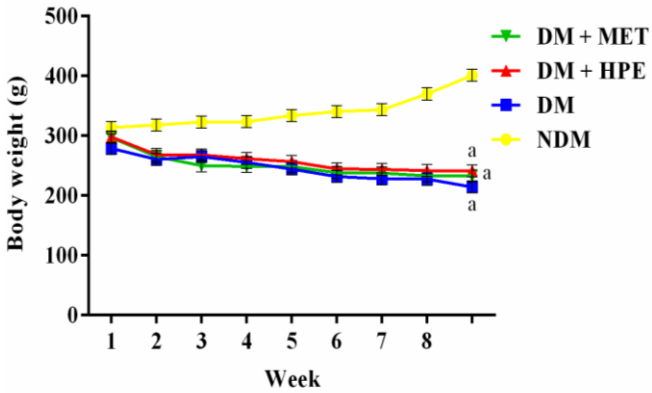


Figure 1a Changes in body weight in all experimental groups
[a] $p<0.05$ vs. NDM group

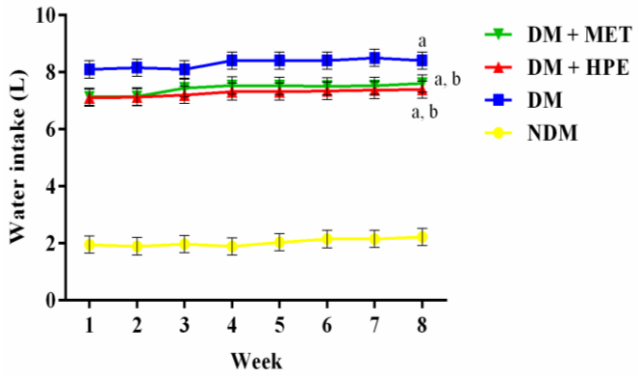


Figure 1b Changes in water intake in all experimental groups [a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group

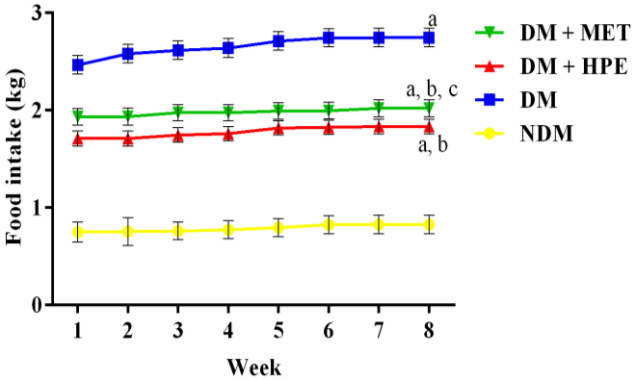


Figure 1c Changes in food intake in all experimental groups [a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group, [c] p<0.05 vs. DM+HPE group

3.2 Effect of HPE Treatment on Blood Glucose, Lipid Profile, and Systemic Blood Pressure

As shown in Figure 2, treatment with HPE and metformin significantly lowered (p<0.05) fasting plasma glucose level. HPE was able to reduce the glucose level in the diabetic rats and this effect is possibly due to its ability to induce β-cell regeneration and thus increase the insulin secretion [27]. Past study showed that aqueous extract of roselle inhibited the carbohydrate-hydrolyzing enzyme (a-amylase and a-glucosidase) [28] resulted in delayed carbohydrate digestion, reduced glucose absorption and consequently control the hyperglycemia [29]. It is suggested that HPE also act as an antioxidant to scavenge the generated ROS from further induced β-cell damage [27].

Deficiency of insulin leads to inactivation of lipoprotein lipase which caused the increased mobilization of free fatty acids from peripheral deposits leading to dyslipidemia [30]. In this study, HPE treatment significantly restored (p<0.05) the total cholesterol, triglycerides, and HDL level in diabetic rats as shown in Figure 3a, Figure 3b and Figure 3c. This is in line with Peng *et al.* [13] which reported a significant reduction in total cholesterol, triglycerides, free fatty acids and (LDL/HDL) ratio in HPE treated rats. The improvement in lipid profile probably due to the inhibition of cholesterol absorption, interference of lipoprotein production and increment in hepatic LDL receptors expression which consequently increase blood LDL removal and cholesterol degradation [31, 32]. Meanwhile, pectin, one of bioactive compound in HPE is found to increase cholesterol degradation to bile acids and decrease triglycerides in serum by enhancing the activity of lipoprotein lipase in adipose tissues [31].

At the end of the eighth week, DM group exhibited significantly higher systolic and diastolic blood pressure (p<0.05) as compared to NDM group (Table 1) which then normalized by HPE and metformin treatment. It was suggested that the hypotensive action of HPE is probably through the promotion of diuresis and ACE inhibitors activity which mediated by the action of delphinidin and cyanidin-3-O-sambubiocides [33, 34].

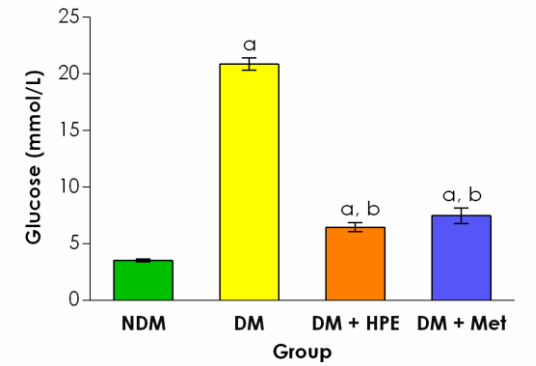


Figure 2 Plasma fasting glucose level in all experimental groups after eight weeks of treatment [a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group

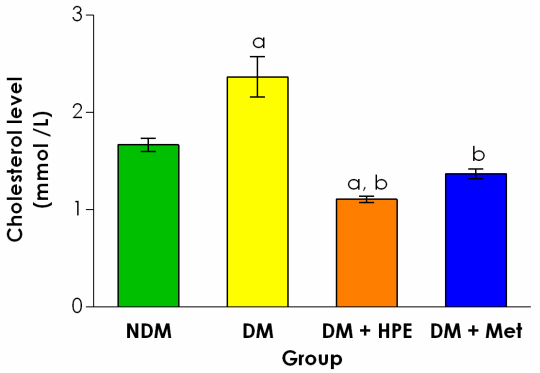


Figure 3a Plasma total cholesterol level in all experimental groups after eight weeks of treatment [a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group

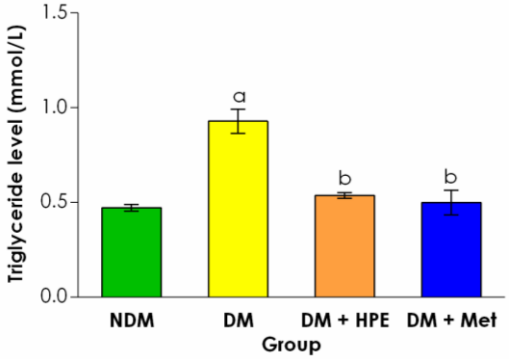


Figure 3b Plasma triglyceride level in all experimental groups after eight weeks of treatment [a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group

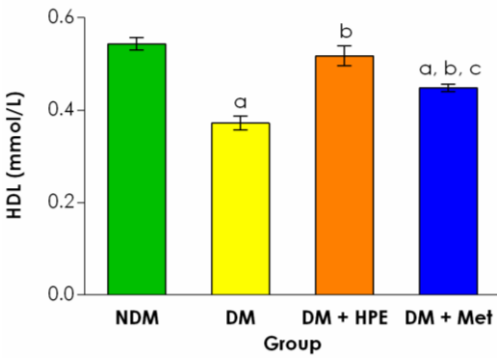


Figure 3c Plasma high-density lipoprotein (HDL) level in all experimental groups after eight weeks of treatment
[a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group, [c] p<0.05 vs. DM+HPE group

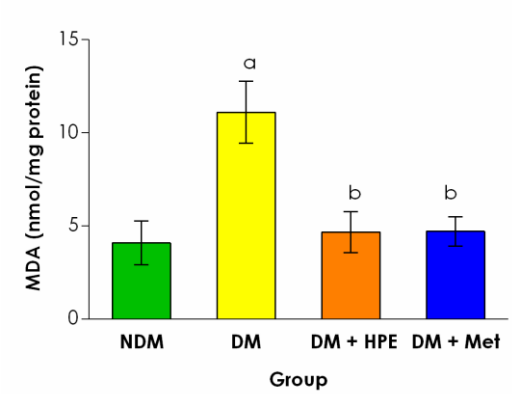
Table 1 Blood pressure in all experimental groups after eight weeks of treatment

GROUP	SBP (mmHG)	DBP (mmHG)	MAP (mmHG)
NDM	129.75±9.74	86.57±4.10	115.97±8.38
DM	172.93±2.12[a]	138.50±5.23	144.33±7.90[a]
DM + HPE	119.67±2.40[b]	95.56±2.93[b]	103.22±1.83[b]
DM + MET	112.67±5.50[b]	91.33±2.39	98.17±2.86[b]

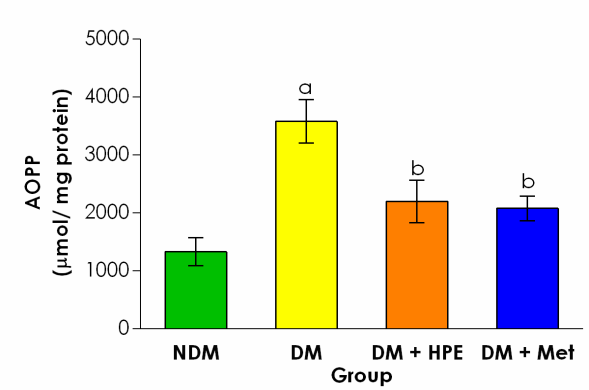
SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. The values are expressed as mean ± SEM
[a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group

3.3 Effect of HPE Treatment on Vascular Oxidative Markers and Antioxidant Status

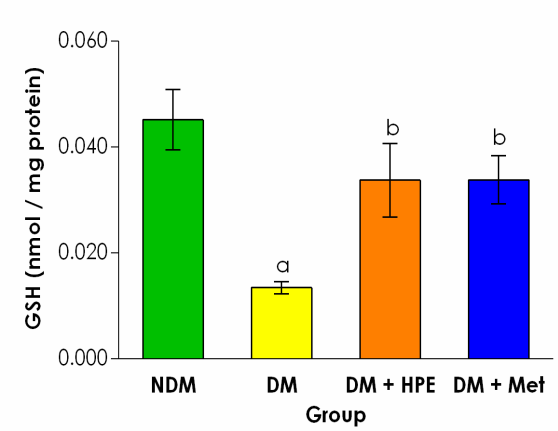
In the present study, the aorta of DM group showed increased in oxidative stress markers (MDA and AOPP) (Figure 4a and Figure 4b) and reduced in GSH level (Figure 4c). HPE treatment significantly (p<0.05) reduced the MDA and AOPP levels as well as increased GSH level. In hyperglycemia condition, increase production of ROS especially superoxide would increase NADPH oxidase activity and eNOS uncoupling which resulted in endothelial dysfunction and oxidative vascular damage [35, 36]. A study by Tseng *et al.* [37] found that roselle contains protocatechuic acid which acts as an antioxidant and thus decrease the formation MDA and AOPP. In addition to protocatechuic acid, it was also reported that anthocyanin pigments contained in roselle can terminate free radical reaction [38]. The polyphenolic compounds in HPE might also able to increase GSH level or directly scavenge the free radicals thus balancing the oxidative stress status in the rats. However, there were no significant differences in the SOD and CAT levels of the aorta (Figure 4d and 4e) among all groups.



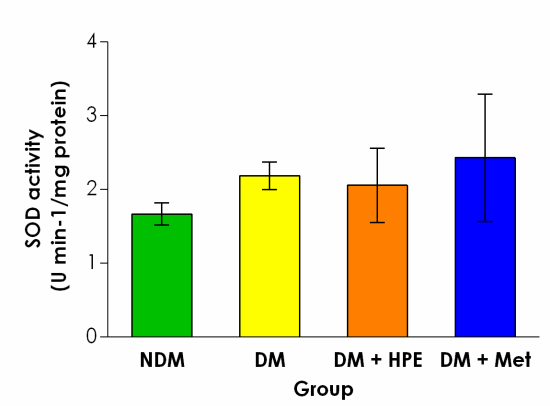
Figures 4a MDA level of aorta in all experimental groups after eight weeks of treatment.
[a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group



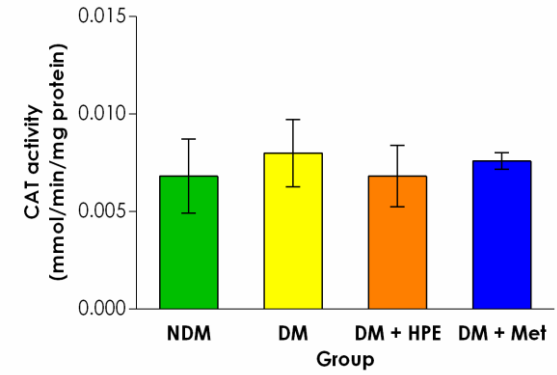
Figures 4b AOPP level of aorta in all experimental groups after eight weeks of treatment
[a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group



Figures 4c GSH level of aorta in all experimental groups after eight weeks of treatment.
[a] p<0.05 vs. NDM group, [b] p<0.05 vs. DM group



Figures 4d SOD activity of aorta in all experimental groups after eight weeks of treatment.



Figures 4e CAT activity of aorta in all experimental groups after eight weeks of treatment.

3.4 Histological Observations of Aorta

Under light microscopic observation, the aorta in all diabetic groups did not show morphological changes as compared to NDM group (Figure 5). The layer of intima, media, and adventitia can be clearly seen in all groups, with the presence of regular elastic fibers and regularly arranged smooth muscle cells in the media layer. The vascular remodeling in eight weeks of diabetic rats can only be observed using transmission electron microscope and no atheroma plaque can be observed at this stage [39].

In diabetic condition, abnormal vascular remodeling is an important key contributing factor to the initiation of hypertension and atherosclerosis. A study conducted by Si *et al.* [40] reported that roselle aqueous extract supplementation is capable to reverse hypertension-induced vascular remodelling as shown by restored intimal media thickness and elastic lamellae count. Another study also showed that roselle able to lower the production of vascular adhesion molecules and oxidation of LDL [41] hence reduce atheroma plaque formation in diabetic condition. It was also proven that vascular remodelling in diabetes can be reversed by reducing the oxidative stress and increasing the antioxidant status [42]. Hirunpanich *et al.* [11] demonstrated that calyx roselle extract exerts an antioxidant effect on LDL oxidation induced by $CuSO_4$. It has been suggested that HPE has the potential to act as an atheroprotective agent due to its bioactive compound such as alkaloids, L-ascorbic acid, arachidic acid, β-carotene, β-sitosterol, citric acid, galactose and pectin as described by Gaet [43].

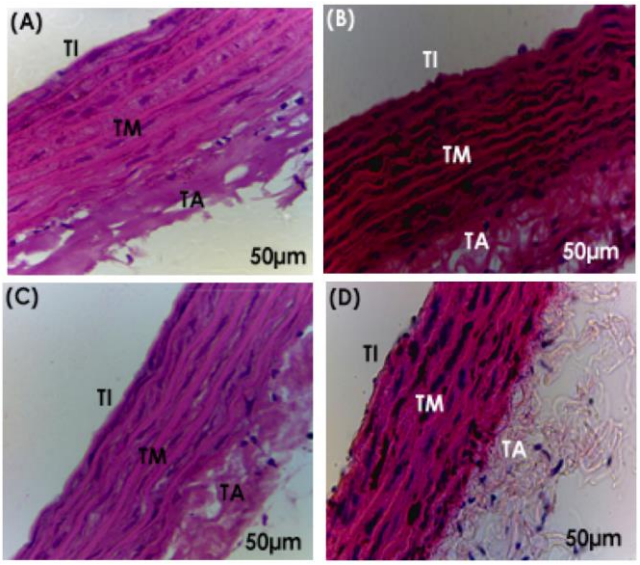


Figure 5 Photomicrograph showing the section of aortic tissues in **(A)** NDM group supplemented with normal saline **(B)** DM supplemented with normal saline **(C)** DM+HPE group supplemented with 100mg/kg HPE and **(D)** DM+MET treated with 150mg/kg metformin where **TI**: tunica intima, **TM**: tunica media, **TA**: tunica adventitia under Hematoxylin and Eosin stain. (LM ×200)

4.0 CONCLUSION

The present findings show that HPE improved blood glucose, blood pressure, dyslipidemia and oxidative stress of the aorta in diabetic rats. Treatment with HPE might able to prevent the oxidative damage and the progression of vascular wall changes, hence reduce the risk of diabetic vascular complications.

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