

Hibiscus sabdariffa Linn. improves the aortic damage in diabetic rats by acting as antioxidant

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ABSTRACT

Chronic hyperglycaemia produces excessive free radicals which could lead to diabetic complications. *Hibiscus sabdariffa* Linn. is proven to have high antioxidant and antihyperlipidemic properties. The aim of the study was to investigate the effects of *H. sabdariffa* polyphenol-rich extract (HPE) in the aorta of diabetic rats. Twenty-four male rats were randomly divided into three groups, i.e. non-diabetes mellitus (NDM), diabetes mellitus (DM) and diabetes mellitus with HPE (DM + HPE). HPE at 100 mg/kg or saline as control vehicle were administered daily through oral gavage for 28 days prior to sacrifice and the aorta was isolated for further analysis. The glucose level in DM + HPE was significantly lower compared to DM, indicating its potential as an antidiabetic agent. Conversely, catalase (CAT), superoxide dismutase (SOD) activity and the level of glutathione (GSH) in DM + HPE were significantly higher than DM. The level of malondialdehyde (MDA) and protein carbonyl were significantly lower in DM + HPE than DM. Lipid profile analysis demonstrated a significant decrease in triglyceride, total cholesterol and LDL-C in DM + HPE compared to DM, however, HDL-C was significantly increased. Microscopic observation showed a disrupted morphology of the thoracic aorta in diabetic rats. HPE may exert protective effects by acting as an antidiabetic, antihyperlipidemic and antioxidant agent to the thoracic aorta in diabetic rats.

INTRODUCTION

Diabetes mellitus (DM) is a chronic metabolic disorder in which prevalence has been growing steadily in our society. This disease occurs when the body could not produce sufficient insulin hormone or when this hormone does not work effectively (Beckman et al., 2013). Poorly managed diabetes leads to serious complications affecting different organs, such as neuropathy, nephropathy, and retinopathy (Inzucchi et al., 2010). Eventually, atherosclerosis would rise as a major threat to the macro vasculature problem which lead to morbidity and mortality in diabetic patients (Dokken, 2008).

Oxidative stress is a common feature of diabetes mellitus (DM), in which the activity of the antioxidant system is over-

whelmed by excessive reactive oxygen species (ROS) production (Valko et al., 2007). Chronic hyperglycaemia increases the generation of free radicals through the activation of polyol pathway, formation of advanced glycosylation end products, activation of protein kinase C and hexosamine pathways (Brownlee, 2005). Oxidation of lipids, proteins and other macromolecules such as DNA occurs during the development of diabetes and during the subsequent complications (Brownlee, 2005). Antioxidant defence mechanisms involve both enzymatic and non-enzymatic strategies. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH). As for common non-enzymatic antioxidants, these include the vitamins A, C and E as well as cofactors like folic acid, uric acids and vitamins B1, B2, B6 and B12 (Maritim et al., 2003).

Hibiscus sabdariffa Linn. or Roselle from the Malvaceae family is one of the most common flowering plant grown in Malaysia. It has high nutritional values especially in the leaves, calyx parts and seeds. The leaves, calyx and seeds are known for

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its high nutritional values with the calyx commonly being used in the preparation of various drinks, jam and jellies (Lin *et al.*, 2012). It is also rich in organic acids such as citric acid, ascorbic acid, pectin and polyphenols which include anthocyanin, phenolic acids, flavonoids and others (Lin *et al.*, 2012). Previous studies have shown that Roselle has great potential as antioxidant (Mos-salam *et al.*, 2011), nephroprotective (Olalye & Rocha, 2007), hypolipidemic (Joshi & Parle, 2006), anti-obesity (Herranz-Lopez *et al.*, 2012) and antihypertensive (Mahadevan *et al.*, 2009). This present study is aimed to observe the effects of *Hibiscus sabdariffa* Linn. polyphenol-rich extract (HPE) in the thoracic aorta of streptozotocin (STZ)-induced diabetic rats.

MATERIALS AND METHODS

Plant extraction

Hibiscus sabdariffa Linn. calyces (specimen voucher: UKMB 40308) was procured from Ai Agro Marketing, Terengganu, Malaysia. About 100 grams of dried ground *H. sabdariffa* Linn. calyx were extracted with 50 mL of an HPLC-grade methanol 99.9% for analytical grade (Thermo Fisher Scientific, UK) and stirred in a 60°C water bath for 30 minutes. The extract was filtered through a Whatman No. 4 filter paper and the residue was extracted twice using similar procedure as above. The extracts were pooled and evaporated using a rotary evaporator (model R-200, Buchi, Switzerland). Residues were solubilized in 200 mL of distilled water and the resulting aqueous solution were partitioned successively with n-hexane and ethyl acetate. Using a rotary evaporator (model R-200, Buchi, Switzerland), the ethyl acetate soluble fraction was evaporated to dryness. The *H. sabdariffa* Linn. polyphenol-rich extract (HPE) was stored at -20°C for further use.

Animal work

Twenty-four male *Sprague-Dawley* rats weighing 250-300 grams were obtained from Laboratory Animal Resources Unit, Universiti Kebangsaan Malaysia (UKM). These rats were placed in polypropylene cages (2 animals per cage) and kept under standard laboratory conditions with a 12 hours' light/dark cycle. The rats also were fed with commercial pellet diet and allowed free access to water *ad libitum* throughout the experiment. Animals were acclimatized to the experimental condition for 1 week prior to experiment and all procedures with animals were strictly adhered to the guidelines of Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC). The animal ethic approval number was FSK/2015/BALKIS/11-FEB./643-FEB.-2015-FEB.-2018.

Induction of diabetes

Diabetes was induced through a single intraperitoneal injection of freshly prepared streptozotocin (STZ) (60 mg/kg) in

overnight fasted rats (Bagri *et al.*, 2009). After 72 hours, fasting blood glucose level were measured using glucometer (ACCU-CHEK® Performa, Roche Diagnostics). Rats with blood glucose levels above 15 mmol/L were considered to be diabetic and selected to be used in this research.

Experimental design

The animals were randomly divided into three groups ($n = 8$ per group), which are the non-diabetics control (NDM), diabetes mellitus (DM) and diabetes mellitus supplemented with HPE (DM + HPE). NDM and DM group received 0.9% NaCl (BDH Laboratory Supplies, UK), while for DM + HPE group. HPE was administered through oral gavage at 100 mg/kg daily for 28 consecutive days (Ramalingam *et al.*, 2016). HPE was re-dissolved in distilled water before used (Lim *et al.*, 2017). Blood glucose level and body weight were recorded weekly throughout the study.

Sample preparation

At the end of the experiment period, the rats were fasted overnight and blood was drawn via orbital sinus, which then sacrificed and thoracic aorta was isolated. The upper third of the thoracic aorta was taken for electron microscopic examination and the remaining was used for homogenates preparation as described by previous study (Upston *et al.*, 2001). The homogenate was stored at -80°C for further analysis.

Blood glucose and lipid profile

Fasting blood glucose was determined using glucose oxidase kits (BioSystems SA, Spain), while plasma triglyceride, total cholesterol and high-density lipoprotein (HDL-C) were measured using commercial kits (Teco Diagnostics, Anaheim). Low-density lipoprotein (LDL-C) concentrations were determined using methods described by previous study (Friedewald *et al.*, 1972) and the results were expressed as mmol/L.

Oxidative stress assessment

Malondialdehyde (MDA) is a marker to determine lipid peroxidation (Stocks & Dormandy, 1971) in which MDA reacts to form thiobarbituric acid reactive species (TBARS) chromogen and its absorbance was measured spectrophotometrically (UV 160A, Shimadzu, Japan) at 532 nm. The results were expressed as nmol/g of protein. According to the method described by previous study (Levine *et al.*, 1990), protein carbonyl which is a marker of protein oxidation, was measured spectrophotometrically (UV 160A, Shimadzu, Japan) at 360 nm, based on the reaction of carbonyl compounds with 2,4-dinitrophenylhydrazine (DNPH) (Sigma-Aldrich St. Louis, USA). The results were expressed as nmol/mg of protein.

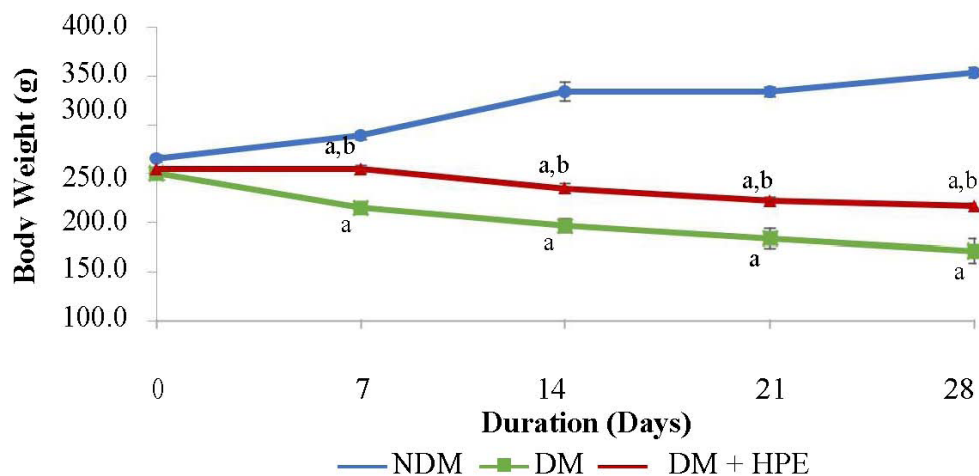


Fig. 1: Body weight between the groups at four weeks of experiment. Values were expressed as mean \pm SEM. Statistical significance for the difference between groups is at $p < 0.05$. ^a $p < 0.05$ compared to NDM; ^b $p < 0.05$ compared to DM at respective week, $n = 8$ for each group.

Antioxidant status

SOD activity was assayed according to protocol mentioned in previous study (Beyer & Fridovich, 1987). The reduction of nitro blue tetrazolium (NBT) (ICN Biomedicals, USA) by superoxide anion formed a purple colour of NBT-diformazan, measured with spectrophotometer (UV 160A, Shimadzu, Japan) at 560 nm, in which one unit of SOD inhibits formation of purple formazan by 50%. The results were expressed as E/mg/min. The activity of CAT was measured according to method described by (Aebi, 1984). Decomposition of H_2O_2 by CAT was measured using spectrophotometer at 240 nm for 3 minutes with 30 seconds interval. The results were expressed as mmol/min/mg of protein. GSH was quantified according to the protocols described previously (Ellman, 1959). GSH oxidation by 5,5-dithiobis-2-nitrobenzoic (DTNB) reagent form 5-thionitrobenzoic (TNB) which is yellow-coloured complex, measured at 415 nm and expressed as mmol/mg of protein.

Transmission electron microscopic evaluation

The microscopic evaluation of the aorta was performed according to the protocol described by previous study (Karasu *et al.*, 1997). Following dissection, the thoracic aorta was cut into small pieces which then fixed at 4°C for 2 hours in 2.5% buffered glutaraldehyde and post-fixed for 90 minutes with osmium tetroxide. Tissues were then dehydrated in ethyl alcohol, followed by propylene oxide, and were embedded in araldite. Semi-thin sections of plastic-embedded samples were stained with toluidine blue, followed by uranyl acetate and lead citrate. The aorta tissues were examined using CM12-Philips transmission electron microscope (Netherlands) equipped with a Mega-View-III digital camera and a Soft Imaging System (Germany) for a computerized acquisition of images.

Statistical analysis

Statistical analysis was done using Statistical Package for the Social Sciences (SPSS) version 23. The data were analysed using one-way analysis of variance (ANOVA), followed by post-hoc Tukey test to estimate the significance differences between groups. Differences were statistically significant at $p < 0.05$. Data were then expressed as mean \pm standard error of mean (SEM).

RESULTS AND DISCUSSION

Effects of HPE on body weight and blood glucose

At the end of the experiment period, both diabetic groups demonstrated significant decrease in body weight ($p < 0.05$) compared to NDM group, however the weight of animals from DM + HPE groups were significantly higher compared to DM groups at $p < 0.05$. (Figure 1). Conversely, the blood glucose level in DM + HPE group was significantly lower ($p < 0.05$) compared to DM group (Figure 2).

STZ-induced diabetes is a well-established diabetic rat model, where the mechanism involves DNA fragmentation, nitric oxide (NO) and free radicals production which then lead to pancreatic β cells damage followed by reduction in insulin secretion (Eleazu *et al.*, 2013). In diabetes, reduced body weight is due to loss of proteins and lipid as a result of proteolysis and lipolysis (Hebert & Nair, 2010). The body weight in diabetic rats was significantly decreased compared to normal rat, as shown in previous study (Almeida *et al.*, 2012). The diabetic rat group showed a consistent higher blood glucose level as expected (Cheng *et al.*, 2013), while aqueous extract of *H. sabdariffa* (HSE) was shown to successfully reduce the blood glucose level significantly probably due to polyphenols are well known as hypoglycaemic agents (Ojuluri *et al.*, 2014; Rosemary *et al.*, 2014).

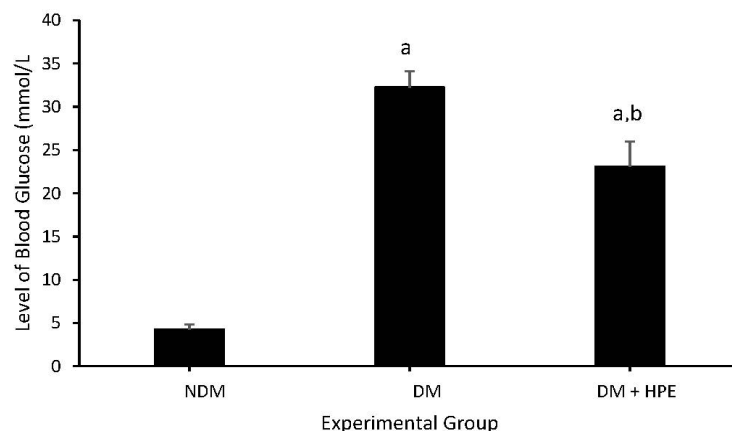


Fig. 2: Level of plasma glucose in NDM, DM and DM + HPE groups after four weeks of study. Data are represented as the mean \pm SEM. Statistical significance for the difference between groups is at $p < 0.05$. ^a $p < 0.05$ compared to NDM group, ^b $p < 0.05$ compared to DM, $n = 8$ for each group.

Table 1: Oxidative stress markers and antioxidant status in NDM, DM and DM + HPE groups after four weeks of study.

Parameters	Group		
	NDM	DM	DM + HPE
TBARS (nmol/mg)	11.38 \pm 1.18	44.56 \pm 4.64 ^a	23.02 \pm 2.42 ^{a,b}
PC (nmol/mg)	3.52 \pm 0.41	27.95 \pm 2.07 ^a	15.48 \pm 0.92 ^{a,b}
SOD (E/mg/min)	957.34 \pm 56.70	230.78 \pm 8.88 ^a	631.31 \pm 30.01 ^{a,b}
CAT (mmol/min/mg)	144.46 \pm 4.28	50.27 \pm 3.93 ^a	78.43 \pm 2.33 ^{a,b}
GSH (mmol/mg)	0.11 \pm 0.01	0.06 \pm 0.004 ^a	0.08 \pm 0.004 ^{a,b}

Data are represented as the mean \pm SEM. Statistical significance for the difference between groups is at $p < 0.05$. ^a $p < 0.05$ compared to NDM group, ^b $p < 0.05$ compared to DM, $n = 8$ for each group.

Effects of HPE on plasma lipid

In diabetic condition, elevation of blood lipid is one of the risk factor for various cardiovascular complications (Rask-Madsen & King, 2013). The present study has shown that STZ-induced diabetes rats causing dyslipidaemia as similarly seen in previous study (Lenzen, 2008). The alteration in blood profile could be due to reduced lipoprotein lipase (LPL) activity secondary to the lowering of plasma insulin levels (Kondo *et al.*, 2007). HPE supplementation prevented the increase of triglyceride, total cholesterol and LDL-C significantly ($p < 0.05$), however it promoted the increment of HDL-C levels in DM + HPE group compared to DM group (Figure 3a-3d). Previously, HSE was shown to reduce serum cholesterol in human (T-L Lin *et al.*, 2007).

Effects of HPE on oxidative stress status of thoracic aorta

The level of MDA and protein carbonyl in both diabetic groups were significantly higher ($p < 0.05$) compared to the NDM group. However, HPE managed to significantly reduce ($p < 0.05$) the level of MDA and PC in DM + HPE group compared to DM group (Table 1). Lipid peroxidation can affect the integrity and membrane permeability as well as inhibit the metabolic processes (Li *et al.*, 2014). Interaction between excessive ROS such as O_2^- and H_2O_2 with polyunsaturated fatty acids (PUFA) has been reported in the aorta of diabetic rat which lead to the production of MDA and therefore cause vascular dysfunction (Paneni *et al.*, 2013). The antioxidant properties in Roselle could significantly

protect lipid peroxidation by promoting antioxidant status in diabetic condition. Diabetic rats supplemented with HSE showed a significantly lower MDA level (Ramalingam *et al.*, 2016) which suggested that Roselle which is rich in polyphenols, such as catechin, may play a role in reducing the levels of MDA by acting as a free radical scavenger (Pandey & Rizvi, 2009).

Free radicals are known to induce protein modification which could also affect cellular functions, mechanisms of signal transduction, enzymatic activity, thermal stability, proteolysis and aging acceleration process (Mohammed *et al.*, 2015). In this study, protein carbonyl, a marker of protein oxidation, was elevated in diabetic rats as supported by previous finding (Sushma *et al.*, 2013). Nevertheless, ethanol extracts of HSE supplementation decrease the protein carbonyl level and this is in agreement with the previous study, that Roselle effectively decrease protein oxidation in renal and hepatic tissues of diabetic rats (Farombi & Ige, 2007). In addition, polyphenols in *H. sabdariffa* was shown to reduce oxidative damage of kidney and improve diabetic nephropathy condition (Lee *et al.*, 2009).

Effect of HPE on antioxidant status (SOD, CAT & GSH) of thoracic aorta

Table 1 shows a significantly low activity of SOD and CAT as well as GSH ($p < 0.05$) in the thoracic aorta of DM group compared to NDM group. However, supplementation of HPE managed to restore SOD, CAT and GSH status significantly ($p < 0.05$) compared to DM group.

SOD is an antioxidant enzyme that catalyses the conversion of anion superoxide ($O_2^{\cdot-}$) into oxygen (O_2) and H_2O_2 and CAT completing detoxification process initiated by SOD. The lower activities of SOD and CAT found in diabetic rats is in agreement with the previous studies (Alpsoy *et al.*, 2014; Jemai & Sayadi, 2015). This was probably due to the increment of antioxidant enzyme usage to compensate with the increased production of ROS. Roselle was suggested to increase the antioxidant activity which then lead to reduction of oxidative damage (Mossalam *et al.*, 2011) and this could possibly due to presence of anthocyanin (Peng *et al.*, 2011). This has been proven in the present study that showed significantly higher SOD and CAT activities in diabetic rats supplemented with HPE.

Moreover, diabetic rats also showed a significantly lower in GSH level which is consistent with the previous study (Kumarappan *et al.*, 2012). Decrease in GSH levels could be due to the overused of GSH as an antioxidant in order to cope with oxidative stress that is usually associated with chronic hyperglycaemia. Roselle is known to have rich source of polyphenol (Mercado-Mercado *et al.*, 2015) and it was found to increase γ -glutamylcysteine synthetase (γ GCS) expression. Therefore, it could cause a significant higher GSH level as seen in diabetic rats with HPE supplementation. This mechanism maybe responsible in the synthesis of glutathione thus increased the intracellular GSH content (Moskaug *et al.*, 2005).

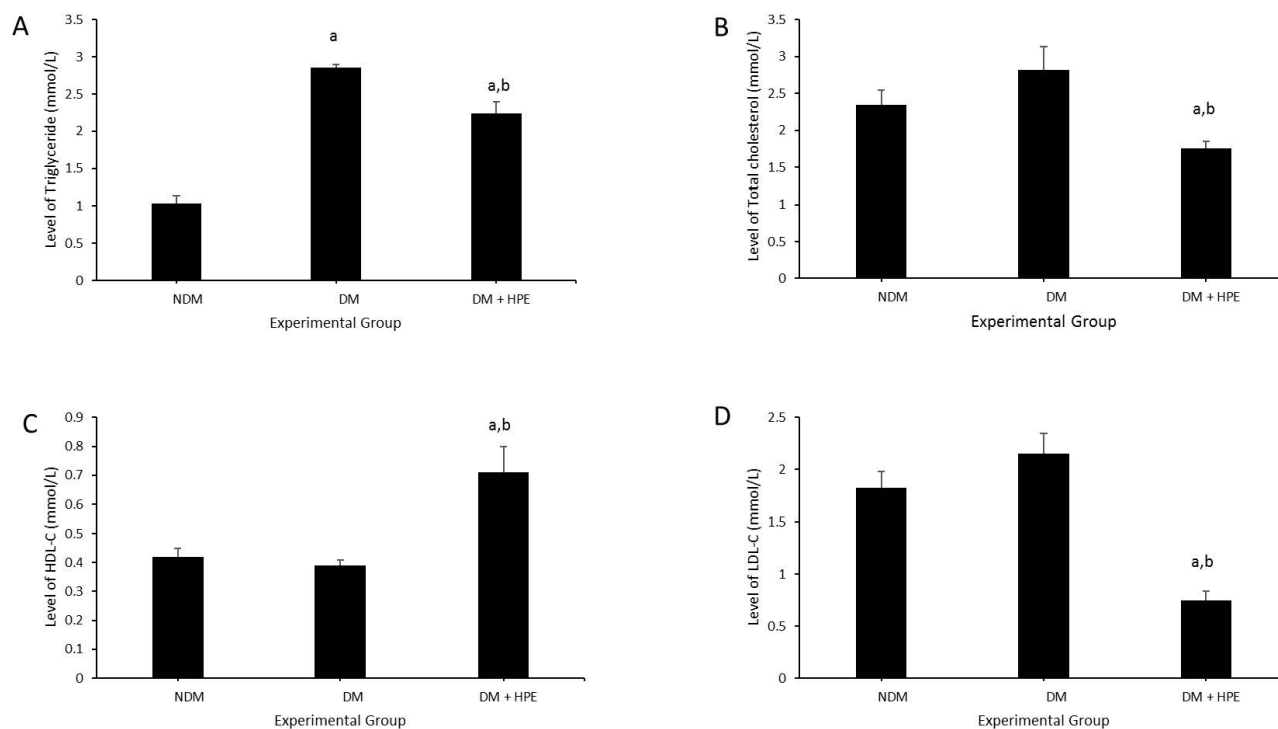


Fig. 3: Level of plasma lipids in NDM, DM and DM + HPE groups after four weeks of study: A) Triglyceride, B) Total cholesterol, C) HDL-C and D) LDL-C. Data are represented as the mean \pm SEM. Statistical significance for the difference between groups is at $p < 0.05$. ^a $p < 0.05$ compared to NDM group, ^b $p < 0.05$ compared to DM, $n = 8$ for each group.

Electron microscopic observation

Observation by electron microscope demonstrated a normal structure of the aorta in NDM rats. A regular feature of vascular smooth muscle (VSMC), endothelial cell, internal elastic lamina and the extracellular matrix was observed (Figure 4A). However, in DM rats (Figure 4B), the disruption of regular aorta feature is shown by intimal thickening, fragmented of VSMC together with atrophy of endothelial cell and irregular feature of tunica intima. It was also evidenced by VSMC proliferation and migration from the tunica media towards tunica intima by the disruption of internal elastic lamina. Excess extracellular matrix with electron-densed amorphous material frequently observed in this condition. HPE treatment in diabetic rats seemed to reduce

the morphological changes by showing a regular appearance of VSMC and endothelial cell together with reduced VSMC proliferation (Figure 4C).

The HPE treatment has been proven to improve blood glucose level and decreased oxidative stress which lead to reduction of ROS production. According to previous study, the antioxidant agent would protect the aorta from further oxidative damage (Rietjens *et al.*, 2007). This condition would then inhibit the ultrastructural changes of aorta thus prevent the development of atherosclerotic lesion as shown in our study. The present study has shown that disrupted aortic ultrastructure has been improved in this finding, in line with previous study that showed endothelium injury impairment by ROS could be restored by antioxidants (Mugge *et al.*, 1991).

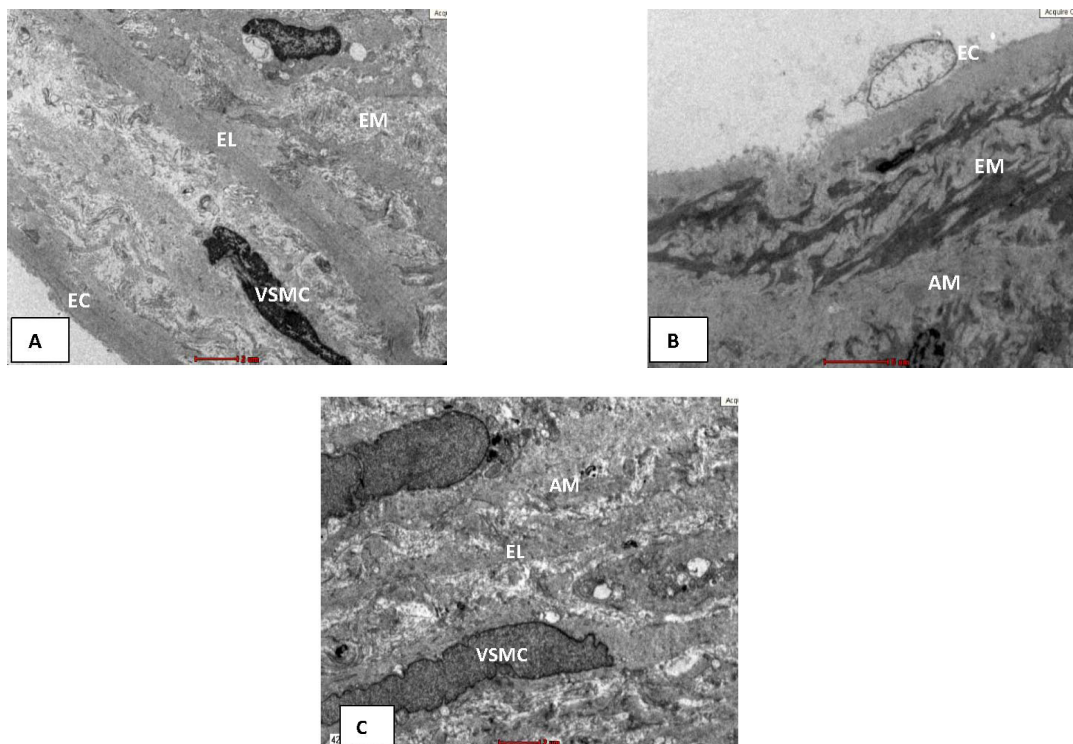


Fig. 4: Electron micrograph of thoracic aorta of rats from NDM groups (A), DM group (B) and DM + HPE group (C) at x4400 magnification. (A) The media layer is composed mainly normal appearance of vascular smooth muscle cells (VSMC), elastic lamina (EL), endothelial cell (EC) and extracellular matrix (EM). (B) Proliferation and fragmentation of VSMC in both the intima and media layer. Fragmented VSMC migrated from the media into the intima, breaking through the EL. The extracellular matrix also consists of amorphous material (AM) in the media layer and the EC became atrophic. (C) The EL and VSMC appeared to be more regular and VSMC did not show proliferation activity.

CONCLUSION

It is concluded that *Hibiscus sabdariffa* Linn. shows potential as an anti-diabetic and anti-hyperlipidaemia by reducing blood glucose levels and improving lipid profile in diabetic rats. It is also shown that HPE decreased oxidative stress markers, and simultaneously increased the antioxidant enzymes, thus preventing oxidative stress and ultrastructure damage in the aorta.

AUTHOR CONTRIBUTIONS

SBB and SZ were the supervisors. NAF, TNT and NLY performed the experiments. NMA wrote and edited the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

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