Original Article

DOI: 10.5582/ddt.2019.01034

# Effects of naringenin on vascular changes in prolonged hyperglycaemia in fructose-STZ diabetic rat model

**Nurul Hannim Zaidun[1], Sahema Zar Chi Thent[1], Mardiana Abdul Aziz[1], Santhana Raj L[2], Azian Abd Latiff[1], Syed Baharom Syed Ahmad Fuad[1,*]**

[1] *Faculty of Medicine, Universiti Teknologi MARA (UiTM), Selangor, Malaysia;*
[2] *Institute of Medical Research, Jalan Pahang, Kuala Lumpur, Malaysia.*

**Summary** **Chronic uncontrolled hyperglycaemia leads to increased oxidative stress and lipid peroxidation resulting in vascular complications and accelerates the progression of diabetic atherosclerosis. Though varieties of modern drugs used in the treatment of diabetes, the complications of diabetes are increasing. Naringenin (NG), has been reported to have potent antioxidant and anti-atherosclerotic properties. However, the effects of NG as vasculoprotective agent in prolonged hyperglycaemia are not well documented. Thus, this study was aimed to determine the effect of NG against vascular changes after prolonged hyperglycaemia in a diabetic rat model. Thirty adult male Sprague-Dawley rats were induced with fructose and streptozotocin to develop the diabetic rat model. After 4 weeks, the rats were randomly divided into 5 groups each group consisting of 6 animals: control, control treated with NG, non-treated diabetes mellitus (DM), DM treated with NG and metformin-treated DM. The treatment with NG (50 mg/kg) and metformin were continued for 5 weeks. The results showed that consumption of NG at 4 weeks post diabetic did not improved blood sugar, blood pressure and serum lipid profile. However, NG did significantly improve oxidative stress parameters in the aortic tissue like malondialdehyde (MDA). Analysis through light microscopy and transmission electron microscope (TEM) reverted the histological changes caused by prolonged hyperglycaemia. The findings thus demonstrated that introduction of NG after prolonged exposure to hyperglycaemia improved the vascular deterioration in diabetic group by decreasing oxidative stress evident by the reduced in the lipid peroxidation activity. Thus, this study showed the potential use of NG as adjunct in managing the diabetic condition during late presentation.**

***Keywords:*** Diabetes Mellitus (DM), naringenin, vascular complications, malondialdehyde (MDA), transmission electron microscope (TEM)

## 1. Introduction

The global prevalence of diabetes mellitus (DM) in 2017 has reached 425 millions and this figure is expected to rise in the coming years (*1*). Chronic uncontrolled hyperglycaemia leads to macrovascular and microvascular complications of diabetes such as vasculopathy. Vascular changes related-DM causes increase morbidity and mortality that significantly contributes to the overall health economics and productivity of a country (*2*).

The pathogenesis of diabetic complications is complex (*3*). Many hypothesized aetiologies had attributed it to the disease process including overproduction of free radicals (*4*). The imbalance between oxidant and antioxidant reactions produces excessive reactive oxygen species and reactive nitrogen species thus increase in oxidative stress. Exposure of vascular wall to the free radicals in chronic hyperglycaemia causes injury to the endothelial cells. Chronic hyperglycaemia also causes reduction in the circulating nitric oxide (NO) secreted by the endothelial cells which triggers endothelial injury by altering the

**Address correspondence to:*
Dr. Syed Baharom Syed Ahmad Fuad, Faculty of Medicine, Universiti Teknologi MARA (UiTM), Sungai Buloh Campus, 47000 Selangor, Malaysia.
E-mail: sbsaf11@gmail.com

vascular tone (*5*) and increased peroxynitrite, a potent free radical derived from nitric oxide (*6*).

Dyslipidaemia as a result of chronic DM has contributed to the development of atherosclerosis in diabetics (*7*). Increased thickness of tunica intima due to endothelial injury is further enhanced by increase in the low density lipoprotein (LDL) and reduction in the high density lipoprotein (HDL) levels in diabetes. Thickening of both tunica intima and media in type 2 diabetes mellitus (T2DM) leads to increased cardiac output which later can be resulted in hypertension (*8*), in addition to the impaired vascular tone secondary to the reduced availability of circulating NO.

Over the years, modern drugs have been the mainstay of treatment for DM for example, in T2DM, metformin remains as one of the first line drugs in treating DM. Nevertheless, the side effects which include lactic acidosis from metformin can be fatal to patients especially the elderly (*9*). Newer antidiabetic drugs such as thiozolidinedione is also associated with increased risk of developing cardiac disease (*10*). Despite the availability of various antidiabetic drugs, the complications of DM is still increasing (*11*). Furthermore, untoward side effects of the drugs warrant an alternative treatment that must be explored.

Naringenin (NG), one of the flavonoid which is found in plants and citrus fruits, has been studied widely in due to its potential actions against diabetes (*12*). NG is found in daily local diet in Malaysia and possesses multiple therapeutics properties such as anti-hypertensive, anti-inflammatory (*13*), anti-dyslipidaemia (*13*), anti-atherosclerotic (14) and anti-oxidative (*15*). Effects of NG in diabetic vessels has also been investigated. For an example, Bei Ren *et al.* had showed that NG improved glucose and lipid metabolism and as well as ameliorated endothelial dysfunction in type 2 diabetic rat model when given 1 week after confirmation of DM (*16*). Many other studies have also investigated the effects of NG on various organs and reported positive outcomes when NG treatment was started immediately from the time of DM development. Nevertheless, its effect on the diabetic vessels after prolonged exposure to uncontrolled hyperglycaemia is not known. Therefore, the aim of the study is to investigate the effects of NG on the diabetic vessels in prolonged (4 weeks) uncontrolled diabetic.

## 2. Materials and Methods

### 2.1. *Animals*

This study was approved by Universiti Teknologi MARA Animal Ethics Committee. Thirty ($n$ = 30), Sprague-Dawley rats weighing 200-250 g were used. Each rat was caged individually in a controlled temperature and humidity. The temperature was set between 25-28°C with 12 h light/dark cycle. The rats were fed with commercially available rat chow diet (Gold coin, Malaysia) and drinking water a*d libitum*.

### 2.2. *Research framework*

After 1 week of acclimatization, rats were randomly divided into control ($n$ = 12) and experimental groups ($n$ = 18). Weight, blood pressure and random blood glucose were measured as baseline values. In experimental group, all the rats were induced with diabetes according to the method described by Wilson *et al*. in 2012 (*17*). Briefly, 10% of fructose was given to the experimental group of rats in drinking water for 2 weeks and then, a single intramuscular injection of 40 mg/kg body weight of streptozotocin (STZ) (98% from Santa Cruz, USA) diluted in citrate buffer (*18*) was given. Control groups received normal drinking water and was injected with citrate buffer as placebo. Three days following STZ injection, a random blood glucose (RBG) was measured using glucometer (Oncall-plus, Germany) at the tail vein. Rats with RBG level of more than 16.5 mmol/L was considered as diabetics (*19*). After 4 weeks of DM induction, the rats were further subdivided into 5 groups; control (Ctr) and control-treated with naringenin (Ctr-NG), non-treated diabetic (DM-Ctr), diabetic treated with metformin (DM-MTF) as positive control group and diabetic treated with naringenin (DM-NG). Weight, blood pressure and random blood glucose were measured prior to treatment as pre-treatment values. The treatment was continued for 5 weeks using oral gavage. The dose of NG 50 mg/kg (*20*) and metformin 150 mg/kg (*21*) were used in the present study. One day before the end of the experiment, weight, random blood sugar and blood pressure were measured as post-treatment values. At the end of the experiment, the rats were fasted overnight and anaesthesized with diethyl ether. Cardiac puncture was performed to collect the blood for fasting lipid profile. Overdose diethyl ether was used for euthanisation. The aorta were harvested from the rats for further biochemical and histological examinations.

### 2.3. *Blood pressure measurement*

Systolic blood pressure (SBP) and diastolic blood pressure (DBP) measurements were taken at after 4 weeks of hyperglycaemia as pre-treatment and at 5 weeks following treatment as post-treatment time. Non-invasive tail-cuffed method was used using CODA system (Kent Scientific, USA). All rats were acclimatised prior to BP measurement by putting the rats in the BP tube 15 min before to the procedure in conscious state. An average of 3 readings were taken for each measurement.

### 2.4. *Measurement of fasting serum lipid profile*

Fasting lipid profile which consists of total cholesterol

(TC), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) were measured at post-treatment and compared between each group. Blood was taken via cardiac puncture following anaesthesia with diethyl ether. The collected blood samples were sent to Gribbles Sdn Bhd Malaysia for further analysis.

2.5. *Collection of tissues*

After 5 weeks of treatment, all rats were euthanised using overdose diethyl ether. Both arch of aorta and thoracic aorta were taken for histological and biochemical analyses, respectively. For the arch of aorta, approximately 2 $mm^3$ of the proximal part was taken for transmission electron microscope analysis.

2.6. *Microscopic analysis*

2.6.1. *Light microscopy*

The arch of aorta was fixed in 10% formaldehyde and all the samples were processed for histological analysis. Tissue was cut using the microtome (Microm, USA) at 5 µm thickness and stained with haematoxylin and eosin for standard histological observation, and alcian blue to observe mucopolysaccharides deposit in the aortic tissues. For histomorphological analysis, the thickness of tunica intima (TI), tunica media (TM) and the ratio between TI and TM were measured by using an image analyzer software (ImageJ, an open resource developed by National Institutes of Health). Four measurements per image were obtained at 0°, 90°, 180° and 270° and the means were calculated (*22*).

2.6.2. *Transmission electron microscopy (TEM)*

Arch of aorta from all rats was cut into 2 $mm^3$ size. The tissues were washed with 0.9% normal saline and fixed with 2.5% glutaraldehyde in phosphate buffer solution (PBS). Then, the tissue was rinsed with distilled water and 2% osmium tetraoxide in distilled water. The tissues were dehydrated using a series of different concentrations of acetone. Infiltration and polymerisation were performed using acetone/resin and pure resin respectively. After polymerization, the specimens were sectioned with a glass knife, stained with toluidine blue solution and further sectioned with diamond knife at 90 nm cut. The specimens were viewed under transmission electron microscope (Tecnai G2 model, FEI, USA).

2.7. *Biochemical analysis*

2.7.1. *Determination of malonaldialdehyde (MDA)*

MDA was measured as oxidative marker in the aortic tissue using a commercial kit (Sigma-Aldrich, German). The assay is based on the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric product which is proportional to the MDA present. Aortic tissue was collected, weigh and was homogenized in 300 mL of the MDA lysis buffer and 3 mL of butylated hydroxytoluene (BHT). The solution was centrifuged at 13,000× g for 10 min. The supernatant (200 µL) from each homogenized sample were placed into a microcentrifuge tube and proceeded to assay reaction. MDA standard solution was prepared according to the manufacturer protocol. MDA standard solution (600 µL) was added into each vial containing homogenised tissue sample. The samples were incubated at 95°C for 60 min and cooled to room temperature in an ice bath for 10 min. Approximately 200 µL of reaction mixture was pipetted into a 96 well plate for analysis. The absorbance was measured at 532 nm (A532) using Perkin Elmer 2030 Multilabel Reader Victor TM X5 machine and expressed in nanomoles per gram of protein.

2.7.2. *Determination of nitric oxide (NO)*

Nitric oxide was determined using Quantichrome Nitric oxide Assay Kits (BioAssay System, USA). Aortic tissue samples (10 mg) were homogenised in phosphate buffer solution and centrifuged at 14,000 rpm at 4°C. The supernatant were taken and deproteinated using 8 µL ZnSO4. The mixture of 150 µL supernatant of sample were vortexed and 8 µL NaOH and vortexed again. The mixture were centrifuged for 10 min at 14,000 rpm. The final 100 µL of supernatant were collected and used for the reaction assay. Working reagent (WR) and standard solution were prepared according to manufacturer protocol. WR was added to every standard solution and samples. The mixture of WR and samples or standards in reaction tubes were incubated for 10 min at 60oC. Then, the reaction tubes were centrifuged and 250 µL of each reaction were separated to the 96 well plate. The optical density (OD) was read at 540 nm using Perkin Elmer 2030 Multilabel Reader Victor TM X5 machine.

2.8. *Statistical analysis*

The data are presented as the means ± standard deviation (SD). Statistical analysis was carried out by using ANOVA followed by Bonferroni post-hoc test. A value of $p < 0.05$ was considered to be significant. All statistical analysis was performed using the SPSS statistical package version 24.0 (SPSS Inc., Chicago, USA).

**3. Results**

3.1. *Confirmation of diabetes*

After induction of diabetes, all rats exhibited clear signs

of overt diabetes in comparison with non-induced rats. The induced rats suffered frequent urination (polyuria) and high fluids and food intake *i.e*. polydipsia and hyperphagia respectively. They were also noticed to become hypoactive and more lethargic, evidenced by the reduced activity upon handling and less movement upon changing of bedding. It was observed that treatment with NG did not improved the morphology, character and behaviour of the diabetic rats. The weights in diabetic rats were not increased in treated and untreated rats as shown in Figure 1.

At post-treatment time (Figure 2), RBG in the DM-Ctr group (27 ± 4.85 mmol/L) remained significantly high ($p < 0.05$) compared to Ctr group (4.98 ± 0.28 mmol/L). Treatment with NG however, did not significantly reduced RBG level in DM-NG (26.7 ± 4.70 mmol/L) group when compared to DM-Ctr group (27 ± 4.85mmol/L). Metformin also did not significantly reduce the level of RBG ($p > 0.05$) but a reducing trend was seen in DM-MTF group (24.43 ± 4.67 mmol/L) compared to DM-Ctr group. Neither hypoglycaemia nor hyperglycaemia was observed at post-treatment time in Ctr-NG group compared to Ctr group.

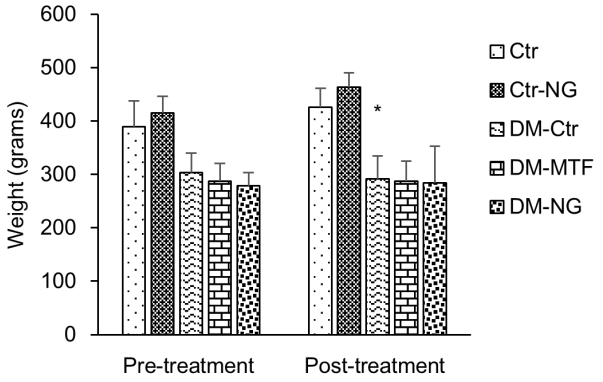


**Figure 1. The changes of weight of the rats at different interval.** (*) significant weight loss was seen in DM-Ctr group compared to Ctr group.

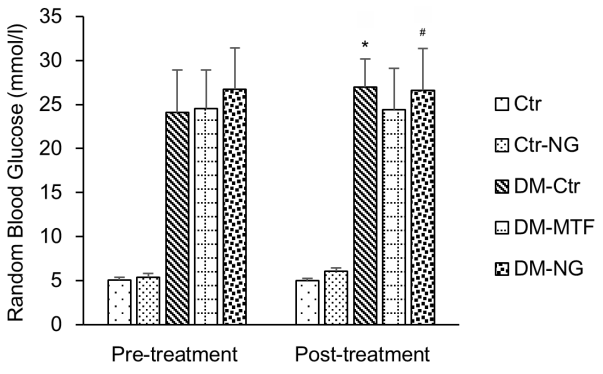


**Figure 2. Effects of NG on RBG level when NG was introduced after 4 weeks of uncontrolled hyperglycemia.** (*) significant increase in RBG, $p < 0.05$, in DM-Ctr group compared to Ctr-group. (#) no significant changes observed in DM-NG compared to DM-Ctr group.

3.2. *Blood pressure*

During pre-treatment time, DM-Ctr group showed significant ($p < 0.05$) changes in SBP (143.78 ± 10.79 mmHg) and DBP (101.00 ± 8.13 mmHg) when compared to Ctr group. However, after 5 weeks of treatment, DM-NG group showed no significant changes ($p > 0.05$) in either SBP (130.17 ± 19.68 mmHg) and DBP (85.72 ± 13.51 mmHg) when compared to DM-Ctr (SBP: 145.22 ± 7.79 mmHg; DBP: 89.11 ± 20.37 mmHg) group (Figure 3).

3.3. *Fasting serum lipid profile*

Fasting lipid profiles were evaluated following 5 weeks of treatment with NG when it was consumed at 4 weeks post diabetic. There were no significant changes observed in all diabetic rats groups in terms of total cholesterol, triglycerides, low density lipoprotein and high density lipoprotein levels when compared to the untreated diabetic rats (DM-Ctr group) ($p > 0.05$). The lipid profiles of non-diabetic rats treated with NG (Ctr-NG) also showed no significant difference ($p > 0.05$) compared with the untreated non-diabetic rats (Ctr). These finding suggest that consumption of NG at 4 weeks post diabetic for 5 weeks had no significant effect on fasting serum lipid profile in diabetic animals

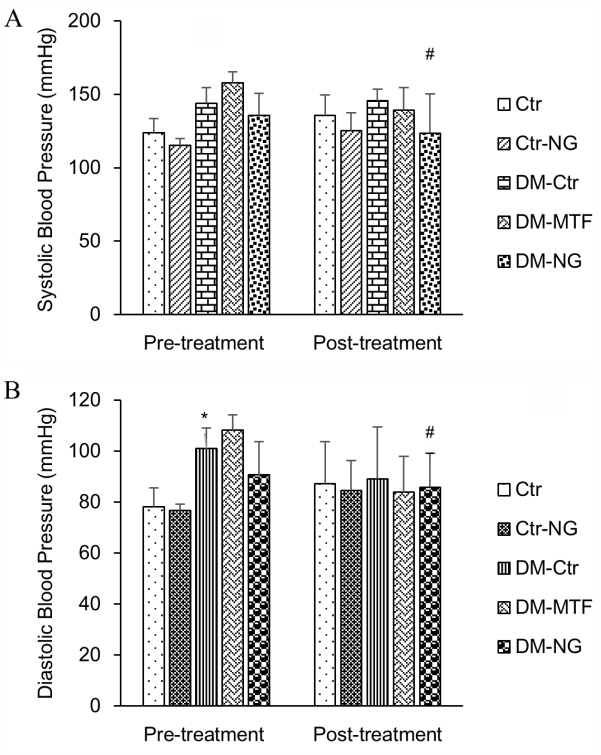


**Figure 3. Changes in the systolic (A) and diastolic (B) blood pressure at different time.** No significant change (#) was observed in systolic and diastolic blood pressure when NG introduced to experimental rats at 4 weeks post diabetic for 5 weeks. (*) significant changes observed in DM-Ctr group compared to Ctr group prior the start of treatment.

**Table 1. Effect of NG when introduced after 4 weeks of diabetes to the fasting serum lipid profile**

| Group | Total Cholesterol (TC) | Triglycerides (TG) | High Density Lipoprotein (HDL) | Low Density Lipoprotein (LDL) |
|---|---|---|---|---|
| Ctr | 1.82 ± 0.26 | 0.805 ± 0.14 | 0.48 ± 0.28 | 0.97 ± 0.09 |
| Ctr-NG | 2.47 ± 0.22 | 0.69 ± 0.11 | 0.305 ± 0.06 | 1.84 ± 0.22 |
| DM-Ctr | 2.64 ± 0.14 | 1.21 ± 0.04 | 0.84 ± 0.32 | 1.27 ± 0.31 |
| DM-MTF | 2.7 ± 0.26 | 0.80 ± 0.04 | 0.89 ± 0.11 | 1.49 ± 0.28 |
| DM-NG | 2.77 ± 0.20# | 1.19 ± 0.29# | 0.97 ± 0.18# | 1.25 ± 0.10# |

(#) showed no significant changes in fasting serum lipid profile in DM-NG compared to DM-Ctr group.

(Table 1).

3.4. *MDA and NO levels of aortic tissue*

MDA level was measured in the aortic tissue (thoracic aorta) as an indicator for lipid peroxidation activity. DM-NG group exhibited significantly decreased ($p < 0.05$) MDA level (0.79 nmol ± 0.02 nmol/mg) compared to the DM-Ctr group (0.84 ± 0.02 nmol/mg). However, the diabetic group treated with metformin (DM-MTF) showed no significant difference compared to DM-Ctr group. No significant changes were observed between NG-treated and untreated non-diabetic rats group (Table 2).

NO level in aortic tissue (thoracic aorta) was measured at the end of the study according to the protocol described by the manufacturer. It was observed that NG-treated DM rats (DM-NG group) showed no significant changes in NO level compared to the untreated group (DM-Ctr) ($p > 0.05$). Similarly, DM-MTF group also showed insignificant change in NO level ($p > 0.05$) compared to control. In the non-DM rats (Ctr and Ctr-NG), treatment with NG did not show any significant changes in NO level (Table 2).

**Table 2. Effect of NG on MDA and NO levels in the aortic tissue when introduced at 4 weeks post diabetic, for 5 weeks.**

| Group | MDA level (nmol/mg) | NO level (μM/mg) |
|---|---|---|
| Ctr | 0.82 ± 0.02 | 30.95 ± 1.26 |
| Ctr-NG | 0.79 ± 0.01 | 38.10 ± 6.12 |
| DM-Ctr | 0.83 ± 0.03 | 35.80 ± 3.72 |
| DM-MTF | 0.82 ± 0.02 | 33.98 ± 3.08 |
| DM-NG | 0.79 ± 0.02* | 37.38 ± 6.47# |

Significant changes were only seen in MDA level (*) but no significant changes were observed in NO level (#) when compared to the DM-Ctr group.

3.5. *Histomorphometry and histological analysis*

In the present study, the thickness of tunica intima (TI) and tunica media (TM) were measured under haematoxylin eosin (H&E) staining which is routine histological staining to observe the morphological feature of a tissue. The measured thickness TI and TM were compared with untreated diabetic group (DM-Ctr) and the ratio between TI:TM were calculated. There was significant increase ($p < 0.05$) in TI thickness in the untreated diabetic rats (DM-Ctr group). Following 5 weeks of treatment with NG, the thickness of TI and TM were reduced in the diabetic rats compared to DM-Ctr group. Similarly, treatment with metformin reduced the TI thickness but not the TM. In addition, there was no significant difference observed in the ratio between TI and TM in all groups (Table 3).

There was loss of endothelial cells and disruption of internal elastic lamina in TI observed in the untreated DM rats on H&E staining (Figure 4). TM elastin was also observed to be less tortuous compared to the untreated non diabetic group. Sublamina area showed high proliferation of vascular smooth muscle cells. After treatment with NG, these distorted morphological features seen in the untreated diabetic rats were lessen, and instead similar features to the non-diabetic rats were observed. The DM-NG group showed presence of normal endothelial cells in TI, prominent internal elastic lamina along with increased in the tortuosity of the elastin. Similar features were also observed in DM-MTF group.

Alcian blue staining was used to stain the connective tissue particularly acid mucopolysaccharides. In the present study, there was increased deposition of acid mucopolysaccharides (blue in colour) in DM-Ctr group (Figure 5). However, DM-NG group showed less deposition of acid mucopolysaccharides along with normal architecture of aortic tissue. Similar

**Table 3. Effect of NG on the thickness of TI and TM when NG introduced at 4 weeks post diabetic**

| Group | Ctr | Ctr-NG | DM-Ctr | DM-MTF | DM-NG |
|---|---|---|---|---|---|
| Thickness of TI (μm) | 8.71 ± 0.65 | 9.71 ± 1.39 | 11.93 ± 1.67 | 9.36 ± 1.04** | 9.04 ± 1.82* |
| Thickness of TM (μm) | 317.58 ± 67.14 | 398.22 ± 59.09 | 470.58 ± 92.12 | 388.48 ± 93.46 | 306.36 ± 85.18* |
| Ratio (TI:TM) | 0.03 | 0.02 | 0.03 | 0.02 | 0.03 |

Thickness of TI and TM was siginificantly reduced in DM-NG group when compared to DM-Ctr group (*). Treatment with metformin in DM-MTF group showed significant reduced in the thickness of TI, but not seen in TM (**).

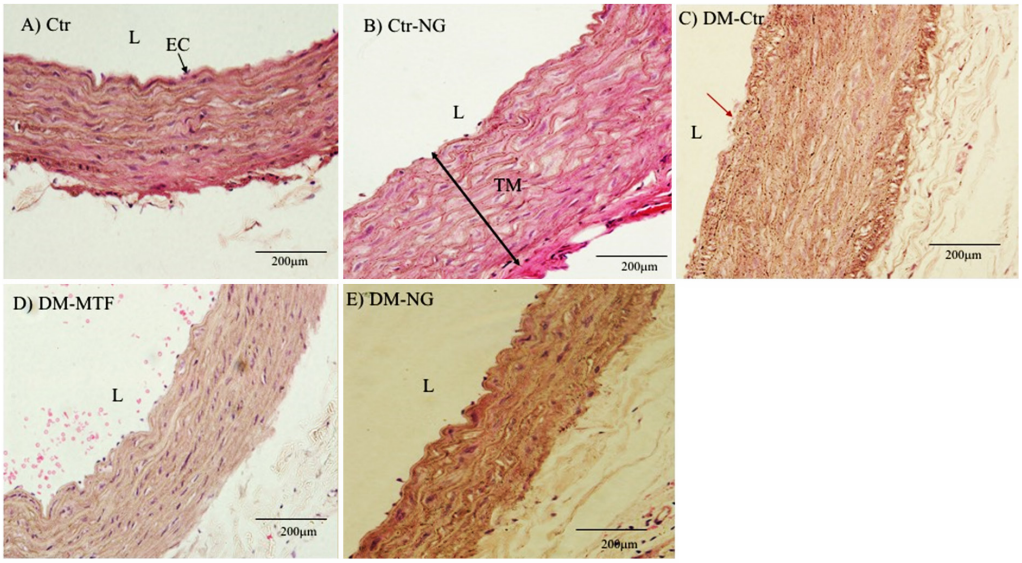


**Figure 4. Photomicrograph of transverse sections of the arch of aorta under H&E staining ×40.** Thicken TI and TM was observed in DM-Ctr compared to Ctr group. Consumption of the NG by the diabetic group in DM-NG after 4 weeks post diabetic showed improvement in the histological features compared to DM-Ctr group. Arrow head (red) showed injury on intimal layer on the DM-Ctr group. TI, tunica intima; TM, tunica media; EC, endothelial cell; L, lumen.

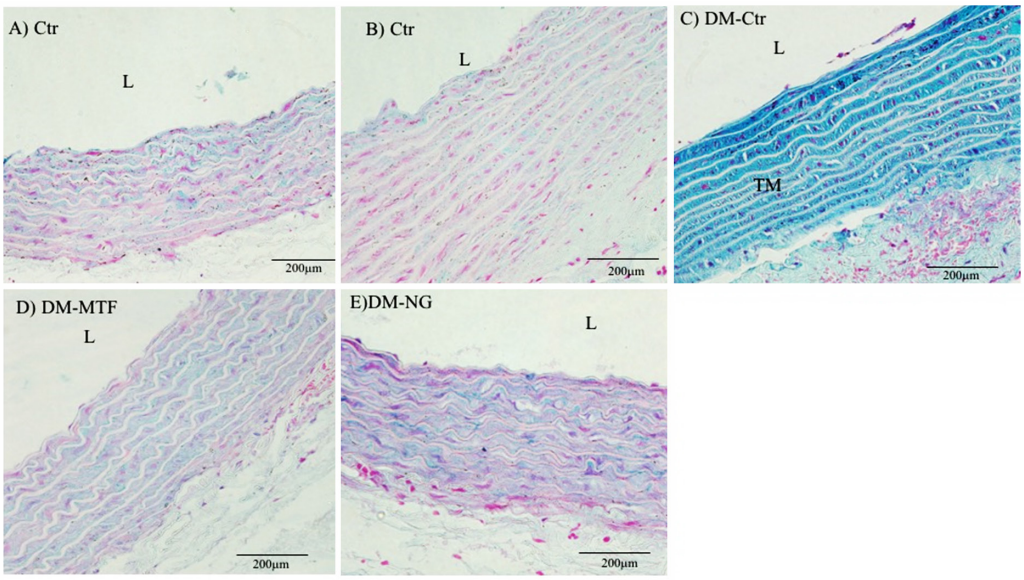


**Figure 5. Photomicrograph of transverse sections of the arch of aorta under Alcian Blue staining ×40.** Increased deposition of acid mucopolysaccharides (blue stain) in both TI and TM was observed in DM-Ctr group compared to Ctr group. In DM-NG group, reduced in mucopolysaccharides was seen compared to DM-Ctr group. TM, tunica media; L, lumen.

features were observed in the DM-MTF group. These histological findings revealed that treatment with NG after 4 weeks exposure to the uncontrolled hyperglycaemia improved the morphological deterioration of aortic tissue in diabetic rats.

### 3.6. *TEM analysis*

The ultrastructural features of endothelial cells in tunica intima, extracellular matrix in the subendothelial region, internal elastic lamina and smooth muscle cells in tunica media were observed under TEM and the results are shown in Figure 6. There were no ultrastructural differences observed in treated and untreated non-diabetic rats (Ctr-NG and Ctr, respectively). Endothelial cells were intact and internal elastic lamina was not disturbed. Smooth muscles cells were normally distributed. However, DM-Ctr group showed some area of desquamation along the intimal region. Retracted endothelial cells were seen and possessed irregular distributions. The notable morphological changes in the intimal layer showed endothelial injury. The appearance normal structure of internal elastic lamina layer was loss. Irregular shaped of smooth muscle

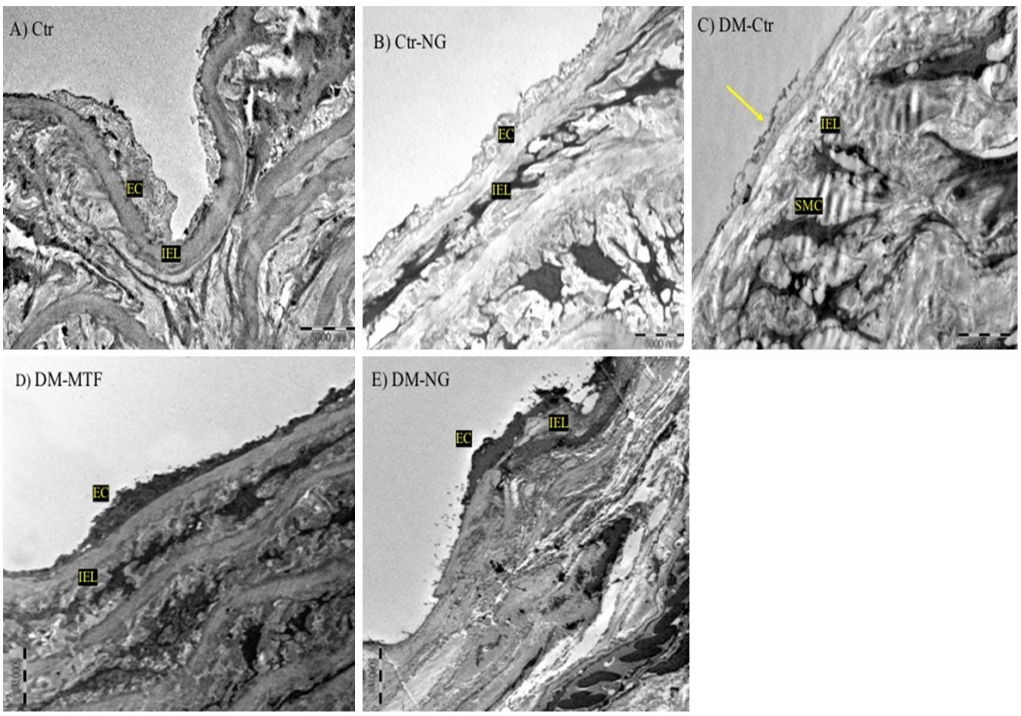


**Figure 6. Micrograph (×8,500) of aorta under TEM.** Noted the disruption of IEL in DM-Ctr compared to Ctr group. Lifting of EC, migration of SMC to intimal layer was noticeable in DM-Ctr which was absent in DM-NG group. EC, endothelial cells; IEL, internal elastic lamina; SMC, smooth muscle cells.

cells was observed in proximity to the internal elastic lamina, migrating to the TI. Nonetheless, DM-NG group showed less ultrastructural deteriorations both in the intimal and media regions. Consumption of NG after 4 weeks of uncontrolled hyperglycaemia improved the diabetic changes. Presence of normal endothelial cells, less disrupted and well-arranged lamina area was observed in DM-NG group. Smooth muscle cells were restored to its normal shape. Similar finding was found in DM-MTF group.

## 4. Discussion

Studies on prolonged hyperglycaemia in diabetic rats has used various duration to define chronic hyperglycaemia. Some studies defined chronic hyperglycaemia as 2 weeks (*23*), 4 weeks (*24*), 8 weeks (*25*) and up to 16 weeks (*26*). In this current study, 4 weeks of prolonged hyperglycaemia is chosen to define chronic DM as no study of NG had been done to investigate the effects of NG on vascular changes in chronic hyperglycaemia. Studies involving the effect of NG on vascular changes had demonstrated NG to effectively prevent abnormal changes in the diabetic vessels through nitric oxide by introducing NG at 1 week post diabetes (*27*). A study on NG by Bei Ren *et al*. had demonstrated NG to significantly regulate glucose and lipid metabolism, and ameliorate vascular dysfunction after introducing NG at 1 week post diabetes in type 2 diabetic rat model (*16*). The longest duration of chronic hyperglycaemia prior the introduction of NG had been reported by Salim *et al*. and Dosari *et al*., where both studies defined 2 weeks as their cut off point prior introducing NG to diabetic group to investigate the effect of diabetic retinopathy and neuropathy (*20,23*). Thus this study was aimed to evaluate the effectiveness of NG at a longer post diabetic period *i.e*. 4 weeks before the introduction of treatment.

The present study used an established diabetic model described by Wilson *et al*. using fructose and low dose STZ (*28*). All the rats in the experimental groups (DM-Ctr, DM-NG and DM-MTF) remained diabetic where the RBG was persistently more than 16.5 mmol/L throughout the experiment prior introduction of NG. The rats were left in non-treated condition for 4 weeks prior starting the treatment. Treatment with NG in the diabetic group did not significantly reduced the blood glucose level. This finding is in contrast to other reported result where immediate treatment with NG on diabetic experimental animal significantly reduced the blood sugar (*29*). Delay in starting treatment had proven to take a longer time to regulate the blood glucose (*30*). Therefore, longer exposure of the cells to the hyperglycaemic condition caused increased in oxidative stress thus more difficult in treating the hyperglycaemic conditions (*31*). Prolonged hyperglycaemia is also known to affect the prognosis and outcome of patients as delay in the intensification of treatment might increase risk in developing cardiac event that could lead to fatality (*32*). In this study, metformin treatment also showed no significant reduction in blood glucose.

This may suggest the possible need for multiple drugs modalities to treat prolonged hyperglycaemia. There is also a need to determine if a higher dose of NG could reduce the blood sugar in chronic state of diabetes.

Complications of DM was reported to result from the imbalance between antioxidant and oxidant reactions. Increased in secondary metabolites due to chronic hyperglycaemia lead to the overproduction of superoxide in the endothelial cells (*7*) thus triggering the oxidative stress in the tissue. Such increase in oxidative stress condition causes endothelial cells injury and promote vascular injury and later on atherosclerosis. On the other hand, NG is one of the flavonoids abundantly found in citrus fruits. It has multiple therapeutics properties against chronic diseases like DM. Apart from being able to improve diabetic condition (*33*), NG is also known to be effective against atherosclerosis accelerated by diabetic condition (*34*). However, in such experiment, NG was being introduced at different intervals from the time of the confirmation of diabetes. In this present study, effects of NG on the general condition including weight, blood pressure, serum lipid profile and histological changes of diabetic aortic tissue in a prolonged diabetic were evaluated.

Immediate introduction of NG to the experimental animal seem to report different results. A study by Sandeep *et al*. (*35*) showed that NG attenuated the loss of weight in the diabetic animal while another study had shown NG to have no significant effect on the weight (*16*). At 2 week post diabetic, NG administration showed no improvement of weight in the diabetic animal (*23*). In this present study, introduction of NG at 4 weeks post diabetic did not significantly affect the weight of the diabetic animal. This result might be due to the condition of the affected animals whereby chronic hyperglycaemia has put the experimental animals into catabolic state. The imbalance between catabolic and anabolic state caused the diabetic rats to lose muscle weight with addition of dehydration hence preventing weight gain.

Blood pressure in diabetic patients tend to rise with prevalence of 30% and 60% for type 1 DM and type 2 DM respectively (*36*). This is partly due to the atherosclerotic process which is accelerated in diabetes (*37*). Consumption of NG in diabetic rats as part of the prevention of hypertension, did not show significant changes on the blood pressure (*14*). However, another study had found that, naringin (aglycone NG) did significantly reduce systolic blood pressure in diabetic animals when it was introduced immediately in metabolic syndrome rat model (*38*). The present study showed that introduction of NG at 4 weeks post diabetic did not significantly improved either systolic or diastolic blood pressure.

Serum lipid profile is commonly affected in diabetes (*39*). Uncontrolled hyperglycaemia causes increased in the glycosylation and oxidation which affect the lipoproteins and contributes to the development of atherosclerosis in diabetes (*36*). Effects of NG on serum lipid profile had shown positive outcomes whereby consumption of NG by the diabetic animal immediately from the time of diagnosis improved serum lipid profile compared to non-treated diabetic animals (*16*). At 10 days post diabetic, consumption of NG by the type 2 diabetic animal model caused alleviation of triglycerides and high density lipoprotein (*40*). However, in this study, 5 weeks of NG consumption by the diabetic animal did not significantly improve lipid profile caused by prolonged uncontrolled hyperglycaemia.

The effects on NG on MDA and NO levels in diabetic aortic tissue were determined. MDA is one of the final products of lipid peroxidation of the unsaturated fatty acids in the cell membrane. It is commonly known as a marker for oxidative stress (*41*). In a diabetic setting, imbalance of antioxidant and oxidant reaction increases the production of MDA. NG is a well-known antioxidant that was shown to reduce lipid peroxidation in the liver, kidney, and pancreas (*15,42*). However, the effects of NG on the level of MDA in the diabetic aortic tissue when the given earlier than 4 weeks post diabetic is not yet available up to the time of writing. In this study, we had found that consumption of NG at 4 weeks post diabetic reduced MDA level in the diabetic aortic tissue. This result supports the existing facts on NG which is not only effective as antioxidant when introduced immediately after the confirmation of diabetes, but may be useful in the managing diabetes when the patients presented late.

NO is secreted by the vascular endothelial cells and acts as key regulator in maintaining the vessel tone (*5,6*). However, NO may interact with superoxide radicals which is increased in hyperglycemia to produce peroxynitrite, a potent oxidant radical. Accumulation of peroxynitrite may trigger endothelial injury and accelerate atherosclerotic process (*43*). *In vitro* studies on NO had shown that NG prevented the decrease in NO level in diabetic cells (*16,44*). Introduction of NG at 1 week post diabetes showed improvement in the availability of NO in the diabetic aorta compared to non-treated diabetic aorta (*27*). Another report showed that consumption of 50 mg/kg body weight of NG after 2 weeks of confirmation of diabetes, had reduced the level of NO in diabetic sciatic tissue (*20*). Similar results was shown in another study where NG introduced at 4 weeks post diabetic showed reduction in the NO level in the diabetic brain tissue (*24*). The present study showed that the effect of NG on the diabetic aortic tissue after consumption of NG at 4 weeks post diabetic to be not significant. Looking at the various reports on the effect of NO when introduced at different interval post diabetes, we can observe the paradox phenomenon of NO in diabetic setting. It was reported that superoxide produced following hyperglycaemia activated other

pathway that caused increased in the expression of inducible nitric oxide (iNOS), thus the net production of NO. However, in chronic hyperglycaemia, more superoxide anions is produced and present in high concentrations. Superoxides interact promptly with newly produced NO to form stronger oxidant which is peroxynitrite and cause decline in the availability of NO in the endothelial cell itself (*45*).

NG that was introduced to the experimental animal at different intervals once the metabolic abnormality was confirmed exhibit positive results in histomorphometrical analysis. For instance, the effect of NG when introduced as supplementation prior the development of the metabolic syndrome rat model had demonstrated improved endothelial injury effect on the vessel wall (*14,34*). Similar result was reported when NG was introduced at 1 week post diabetic, whereby histological analysis by light microscopy and TEM showed less injury to the aorta of the diabetic rats compared to untreated diabetic group (*16*). In this study, consumption of NG for 5 weeks at 4 weeks post diabetic caused significant reduction in the thickness of tunica intima and media with reduced the collagen deposition in the vessel wall of the diabetic aorta compared to the untreated diabetic group. Effect of NG can be clearly seen in TEM even after delayed treatment *i.e.* 4 weeks following the confirmation of diabetes.

In conclusion, effect of NG when introduced at 4 weeks post diabetic, for 5 weeks resulted in reduction of oxidative stress evident by the reduce lipid peroxidation activity in diabetic group. It also improved the histomorphological changes of the aortic tissue in chronic diabetes. These findings are comparable to the effects of NG when it was given immediately after the diagnosis of diabetes. However, the effect of NG as antihyperglycemic and antilipidemic when it was introduced after 4 weeks of uncontrolled hyperglycaemia was not evident in this study compared to the earlier study whereby NG is effectively reduced the blood glucose and improved serum lipid profile when introduced at the beginning of the diabetic disease. Administration of NG after 4 weeks diabetes also did not affect the level of NO in the diabetic aortic tissue. Thus detailed research of NG might be needed as to either increased the dose of NG or longer duration of treatment might be considered for NG to be effective in managing diabetes in delayed presentation.

**Acknowledgements**

This research was fully funded by LESTARI: 600-IRMI/myra5/3/LESTARI (9/2016), Universiti Teknologi MARA (UiTM).

**References**

1. Forouhi NG, Wareham NJ. Epidemiology of diabetes. Medicine. 2014; 42:698-702.
2. World Health Organization. Diabetes country profiles (Malaysia). *http://www.who.int/diabetes/country-profiles/en/#M*. (accessed on 8th April 2019)
3. Forbes JM, Cooper ME. Mechanisms of diabetic complications. Physiol Rev. 2013; 93:137-188.
4. Giacco F. Oxidative stress and diabetic complications. Circ Res. 2011; 107:1058-1070.
5. Zhao Y, Vanhoutte PM, Leung SWS. Vascular nitric oxide: Beyond eNOS. J Pharmacol Sci. 2015; 129:83-94.
6. Pitocco D, Zaccardi F, Di Stasio E, Romitelli F, Santini SA, Zuppi C, Ghirlanda G. Oxidative stress, nitric oxide, and diabetes. Rev Diabet Stud. 2010; 7:15-25.
7. Tomkin G.H., and Owens D. LDL as a cause of atherosclerosis. Atheroscler Thromb J Atheroscler Thromb. 2012; 5:13-21.
8. Nezu T, Hosomi N, Aoki S, Matsumoto M. Carotid intima-media thickness for atherosclerosis. J Atheroscler Thromb. 2016; 23:18-31.
9. Peters N, Jay N, Barraud D, Cravoisy A, Nace L, Bollaert PE, Gibot S. Metformin-associated lactic acidosis in an intensive care unit. Crit Care. 2008; 12:R149.
10. Bolger AF, Herrington D, Eckel RH, Kaul S, Giugliano RP. Thiazolidinedione drugs and cardiovascular risks. Circulation. 2010; 121:1868-1877.
11. Deshpande AD, Harris-Hayes M, Schootman M. Epidemiology of diabetes and diabetes-related complications. Phys Ther. 2008; 8:1254-1264.
12. Priscilla DH, Roy D, Suresh A, Kumar V, Thirumurugan K. Naringenin inhibits a-glucosidase activity: A promising strategy for the regulation of postprandial hyperglycemia in high fat diet fed streptozotocin induced diabetic rats. Chem Biol. 2014; 210:77-85.
13. Nyane NA, Tlaila TB, Malefane TG, Ndwandwe DE, Owira PMO. Metformin-like antidiabetic, cardio-protective and non-glycemic effects of naringenin: Molecular and pharmacological insights. Eur J Pharmacol. 2017; 803:103-111.
14. Mulvihill EE, Assini JM, Sutherland BG, Dimattia AS, Khami M, Koppes JB, Sawyez CG, Whitman SC, Huff MW. Naringenin decreases progression of atherosclerosis by improving dyslipidemia in high-fat-fed low-density lipoprotein receptor-null mice. Arterioscler Thromb Vasc Biol. 2010; 30:742-748.
15. Sharma A, Patar AK, Bhan S. Cytoprotective, antihyperglycemic and antioxidative effect of naringenin on liver and kidneys of Swiss diabetic mice. Int J Heal Sci Res. 2016; 6:118-131.
16. Ren B, Qin W, Wu F, Wang S, Pan C, Wang L, Zeng B, Ma S, Liang J. Apigenin and naringenin regulate glucose and lipid metabolism, and ameliorate vascular dysfunction in type 2 diabetic rats. Eur J Pharmacol. 2016; 773:13-23.
17. Wilson RD, Islam S. Fructose-fed streptozotocin-injected rat : An alternative model for type 2 diabetes. Pharmacol Rep. 2012; 64:129-139.
18. Kim J, Kang M, Choi H, Jeong S, Lee Y, Kim J. Quercetin attenuates fasting and postprandial hyperglycemia in animal models of diabetes mellitus. 2011; 5:107-111.
19. Fernandes AAH, Novelli ELB, Junior AF, Galhardi CM. Effect of naringerin on biochemical parameters in the streptozotocin-induced diabetic rats. Brazilian Arch Biol Technol. 2009; 52:51-59.
20. Al-Rejaie SS, Aleisa AM, Abuohashish HM, Parmar MY, Ola MS, Al-Hosaini AA, Ahmed MM. Naringenin neutralises oxidative stress and nerve growth factor

discrepancy in experimental diabetic neuropathy. Neurol Res. 2015; 37:924-933.
21. Salemi Z, Rafie E, Goodarzi MT, Ghaffari MA. Effect of metformin, acarbose and their combination on the serum visfatin level in nicotinamide/streptozocin-induced type 2 diabetic rats. Iran Red Crescent Med J. 2016; 18:e23814.
22. Abas R, Othman F, Thent ZC. Effect of Momordica charantia fruit extract on vascular complication in type 1 diabetic rats. EXCLI J. 2015; 14:179-189.
23. Al-Dosari DI, Ahmed MM, Al-Rejaie SS, Alhomida AS, Ola MS. Flavonoid naringenin attenuates oxidative stress, apoptosis and improves neurotrophic effects in the diabetic rat retina. Nutrients. 2017; 9:pii: E1161.
24. Rahigude A, Bhutada P, Kaulaskar S, Aswar M, Otari K. Participation of antioxidant and cholinergic system in protective effect of naringenin against type-2 diabetes-induced memory dysfunction in rats. Neuroscience. 2012; 226:62-72.
25. Badalzadeh R, Mohammadi M, Yousefi B, Faranjia S, Najafi M, Mohammadi S. Involvement of glycogen synthase kinase-3ß and oxidation status in the loss of cardioprotection by postconditioning in chronic diabetic male rats. Adv Pharm Bull. 2015; 5:321-327.
26. Davidson EP, Coppey LJ, Shevalye H, Obrosov A, Randy H, Yorek MA. Impaired corneal sensation and nerve loss in a type 2 rat model of chronic diabetes is reversible with combination therapy of menhaden oil, α-lipoic acid and enalapril. Cornea. 2017; 36:725-731.
27. Roghani M, Fallahi F, Moghadami S. Citrus flavonoid naringenin improves aortic reactivity in streptozotocin-diabetic rats. Indian J Pharmacol. 2012; 44:382.
28. Wilson RD, Islam S, Islam MS. Fructose-fed streptozotocin-injected rat: An alternative model for type 2 diabetes. Pharmacol Rep. 2012; 64:129-139.
29. Oršolić N, Gajski G, Garaj-Vrhovac V, Crossed D Signikić D, Prskalo ZŠ, Sirovina D. DNA-protective effects of quercetin or naringenin in alloxan-induced diabetic mice. Eur J Pharmacol. 2011; 656:110-118.
30. Zografou I, Strachan MWJ, McKnight J. Delay in starting insulin after failure of other treatments in patients with type 2 diabetes mellitus. Hippokratia. 2014; 18:306-309.
31. Kalansooriya A, Whiting PH, Haddad F, Holbrook I, Jennings PE. The relationship between chronic glycaemic control and oxidative stress in type 2 diabetes mellitus. Br J Biomed Sci. 2016; 65:71-74.
32. Nichols GA, Romo-LeTourneau V, Vupputuri S, Thomas SM. Delays in anti-hyperglycaemic therapy initiation and intensification are associated with cardiovascular events, hospitalizations for heart failure and all-cause mortality. Diabetes Obes Metab. 2019; 21:1551-1557.
33. Ramprasath T, Senthamizharasi M, Vasudevan V, Sasikumar S, Yuvaraj S, Selvam GS. Naringenin confers protection against oxidative stress through upregulation of Nrf2 target genes in cardiomyoblast cells. J Physiol Biochem. 2014; 70:407-415.
34. Assini JM, Mulvihill EE, Sutherland BG, Telford DE, Sawyez CG, Felder SL, Chhoker S, Edwards JY, Gros R, Huff MW. Naringenin prevents cholesterol-induced systemic inflammation, metabolic dysregulation, and atherosclerosis in $Ldlr^{-/-}$ mice. J Lipid Res. 2013; 54:711-724.
35. Sandeep MS, Nandini CD. Influence of quercetin, naringenin and berberine on glucose transporters and insulin signalling molecules in brain of streptozotocin-induced diabetic rats. Biomed Pharmacother. 2017; 94:605-611.
36. Leon BM. Diabetes and cardiovascular disease: Epidemiology, biological mechanisms, treatment recommendations and future research. World J Diabetes. 2015; 6:1246.
37. Stout RW. Diabetes and atherosclerosis. Biomed Pharmacother. 1993; 47:1-2.
38. Alam MA, Kauter K, Brown L. Naringin improves diet-induced cardiovascular dysfunction and obesity in high carbohydrate, high fat diet-fed rats. Nutrients. 2013; 5:637-650.
39. Schofield JD, Liu Y, Rao-Balakrishna P, Malik RA, Soran H. Diabetes dyslipidemia. Diabetes Ther. 2016; 7:203-219.
40. Ahmed OM, Hassan MA, Abdel-Twab SM, Abdel Azeem MN. Navel orange peel hydroethanolic extract, naringin and naringenin have anti-diabetic potentials in type 2 diabetic rats. Biomed Pharmacother. 2017; 94:197-205.
41. Yoshikawa T, Naito Y. What is oxidative stress? Japan Med Assoc J. 2002; 45:271-276.
42. Annadurai T, Muralidharan AR, Joseph T, Hsu MJ, Thomas PA, Geraldine P. Antihyperglycemic and antioxidant effects of a flavanone, naringenin, in streptozotocin-nicotinamide-induced experimental diabetic rats. J Physiol Biochem. 2012; 68:307-318.
43. Adela R, Nethi SK, Bagul PK, Barui AK, Mattapally S, Kuncha M, Patra CR, Reddy PN, Banerjee SK. Hyperglycaemia enhances nitric oxide production in diabetes: A study from South Indian patients. PLoS One. 2015; 10:1-17.
44. Qin W, Ren B, Wang S, Liang S, He B, Shi X, Wang L, Liang J, Wu F. Apigenin and naringenin ameliorate PKCβII-associated endothelial dysfunction via regulating ROS/caspase-3 and NO pathway in endothelial cells exposed to high glucose. Vascul Pharmacol. 2016; 85:39-49.
45. Wright E, Scism-Bacon JL, Glass LC. Oxidative stress in type 2 diabetes: The role of fasting and postprandial glycaemia. Int J Clin Pract. 2006; 60:308-314.

*(Received May 23, 2019; Revised June 30, 2019; Accepted August 20, 2019)*