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Effects of Naringin on Apoptosis and Oxidative Stress in Type 2 Diabetic Rats

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Abstract. Oxidative stress and apoptosis have been reported to play major roles in the pathogenesis of Type 2 Diabetes Mellitus (T2DM) through insulin resistance and β -cell dysfunction. Naringin is a citrus derived flavonoid that has been reported for its antioxidant properties. Even though effects of naringin in T2DM related oxidative stress has been reported, varying dose concentration in oxidative stress and mechanism of action involving T2DM related apoptosis is far-fetched. This research studied the effects of naringin at varying dose concentration on apoptosis, biomarkers of organ function and oxidative stress in high fat diet/low-streptozotocin-induced T2DM in albino Wistar rats. Diabetic rats were treated with naringin at 50mg/kg, 100mg/kg and 200mg/kg body weight for 21 days. Some biomarkers of organ function and oxidative stress in the animals were assayed using spectrophotometric techniques. The levels of expression of caspases and apoptotic regulators were quantified using semi-quantitative reverse transcriptase polymerase chain reaction (RT PCR). Enzyme-linked immunosorbent assay was used to determine inducible nitric oxide synthase (iNOS) level. Naringin treatment shows a dose dependent significant ($p < 0.05$) reduction in the plasma concentration of γ -glutamyltransferase, alkaline phosphatase and aspartate aminotransferase. Increasing dosage of Naringin significantly ($p < 0.05$) reduced lipid peroxidation, glutathione- s-transferase, glutathione peroxidase and glutathione reductase activities in the liver. Naringin treatment also showed a significant ($p < 0.05$) increase in the expression of caspase 3 and reduction in BCL-2 as against the diabetic control. In addition, there was dose dependent decrease in plasma CO₂ concentration and increase in the plasma iNOS concentration as compared to the diabetic control. This result highlights positive effect of naringin as an antioxidant, its role in apoptosis and also reverting the effects of organ damage in type 2 diabetes.

INTRODUCTION

Type 2 diabetes (T2DM) is a bipolar disease characterized by hyperglycaemia through insulin resistance and insulin secretion defects (1). T2DM is a major public health problem with increasing prevalence with the metabolic disorder estimated by the World Health Organization to hit a prevalence of over 380 million people globally by 2025 [2]. Insulin resistance and β -cell dysfunction in T2DM has been linked to overproduction of reactive oxygen species (ROS), hyperglycemia and lipid accumulation and eventual occurrence of increased apoptosis and loss of insulin integrity [2]. Excess glucose from hyperglycemia also reacts with plasma proteins leading to ROS production through formation of glycation end products [3]. This increase in ROS with concurrent decrease in plasma antioxidant is a major cause of oxidative DNA damage and insulin resistance in diabetic patient [4]. Also, there have been reports suggesting increased apoptosis in T2DM thus building possible relationship between apoptosis, β -cell dysfunction and oxidative stress [5]. Oxidative stress induced by hyperglycemia has been described to activate apoptosis through the intrinsic apoptotic pathway [6].

Complications encountered from the use of synthetic drugs as a form of T2DM have accelerated the use of encouraged the use of natural products like flavonoids. Some of these complications include hypoglycaemia and drug resistance [7]. Flavonoids including naringin have been explored for various properties in treatment of metabolic disorders. Naringin is a flavone glycoside and an aglycone of naringenin that gives grapefruit its bitter taste [8]. Naringin has been reported for its antioxidant property but its role in apoptosis linked T2DM is

unclear. This study, therefore, investigated the effects of naringin on oxidative stress and apoptosis in HFD/low STZ-induced experimental model of T2DM in rats.

MATERIALS AND METHODS

Chemicals

Streptozotocin (STZ) was purchased from Sigma-Aldrich, Missouri, U.S.A while Metformin however was gotten from Swipha Pharma, Lagos, Nigeria. Naringin was obtained from Hunan Kang Biotechnology Company, Hunan Province, P. R China. All other chemicals used in this study were of Sigma-Aldrich, Missouri, U.S.A standard.

Animals

Thirty male albino rats of average weight of 170 ± 20 g were used for this experiment. Animals were housed in an animal room under normal temperature (22 ± 2 °C) with 12 hour light and dark cycle. The animals were allowed to acclimatise for three weeks before the commencement of the study. The experiment approved by the institutional ethical review committee was carried out according to the guidelines of the committee in Covenant University, Ota, Ogun State, Nigeria.

Experimental design

The rats were divided into six experimental groups and maintained on their respective diets for a period of 9 weeks with weight gain recorded weekly. Five of the groups were fed with High fat diet (HFD) with 45% fat while the last group was fed with normal pellet diet.

- Group A, Diabetic Control: Rats fed with HFD.
- Group B, Diabetic rats treated with 50mg/kg Naringin: Rats fed with HFD
- Group C, Diabetic rats treated with 100mg/kg Naringin: Rats fed with HFD
- Group D, Diabetic rats treated with 200mg/kg Naringin: Rats fed with HFD
- Group E, Diabetic rats treated with 150mg/kg Metformin: Rats fed with HFD
- Group N, Normal Control: Rats fed with normal diet.

Animal treatment and sample collection

After four weeks of feeding, the HFD fed groups were administered intraperitoneally with 30mg/kg body weight streptozotocin (STZ) twice in two weeks while the control group was administered the vehicle, 1ml/kg body weight 0.1M citrate buffer (10). Fasting blood glucose was checked a week after the second dose of STZ had been administered. Naringin treatment was then administered for 21 days. Sample collection and preparation was done as described by Rotimi *et al.*, [9].

Determination of Plasma Inducible Nitric Oxide Synthase (iNOS)

Plasma inducible nitric oxide synthase (iNOS) concentration was however determined by enzyme-linked immunosorbent assay (ELISA) using kit from Hangzhou east biopharm, China.

Determination of biomarkers of organ damage

Some biomarkers of organ damage in plasma including urea, creatine kinase, lactate dehydrogenase, γ -glutamyltransferase, α -amylase, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase were assayed spectrophotometrically using kits from BioSino Biotechnology & Science Inc., Changping District Beijing, China.

Determination of Antioxidant enzymes

Antioxidant enzyme activities were assayed using spectrophotometric method. Glutathione reductase was determined using the method of Mavis and Stellwagen [11]. Glutathione peroxidase was determined according to the method of Rotruck *et al.* [12]. Glutathione-S-transferase was determined according to the method of Habig *et al.* [13]. Superoxide dismutase was determined according to the method of Marklund and Marklund [14]. Lipid peroxidation was determined according to the method of Buege and Aust [15] while glutathione concentration was determined according to the method of Ellman [16].

Expression of Hepatic Apoptotic Genes

Template RNA extracted from the liver using the Aidlab EASYspin Plus RNA extraction kit from Beijing, China. Reverse transcriptase polymerase chain reaction (RT-PCR) was carried out using the Transgen *EasyScript* one-step RT-PCR Supermix from China. All reactions were carried out on ice and manufacturer's instructions were duly followed using the method initially described by Rotimi *et al.* [9].

The forward (F) and reverse (R) primers used and their sequences are listed in the table below:

TABLE 1: Gene Specific Primer sequences

Gene	5'-3' Sequence
β-Actin	Forward: GTCAGGTCATCACTATCGGCAAT Reverse: AGAGGTCTTTACGGATGTCAACGT
BCL-2	Forward: TTTTGCTGAGTTACCGGCGA Reverse: GCCACAAGGGTAGCCAGAAT
Caspase 3	Forward: GAGCTTGGAACGCGAAGAAA Reverse: TAACCGGGTGCGGTAGAGTA
Caspase 9	Forward: GCGCGACATGATCGAGGATA Reverse: TCTCCATCAAAGCCGTGACC

Statistical Analysis

Data was analysed using Mean± SEM of five replicates in each group. Analysis of Variance (ANOVA) was carried out to test for the level of homogeneity at $p < 0.05$ among the groups while Duncan's Multiple Range Test (MRT) was used to separate the heterogeneous groups.

RESULTS

Levels of reduced glutathione (GSH), TBARS, glutathione S-transferase (GST), glutathione reductase (GRx) and glutathione peroxidase (GPx) were assayed in the heart, liver, brain, testes and kidney. The levels of TBARS, GST, GRx and GPx were significantly ($p < 0.05$) increased in diabetic control group compared to normal group. Naringin caused a significant ($p < 0.05$) dose-dependent reduction of levels of TBARS, GST, GRx and GPx in all the organs. GSH levels was however higher in the liver, kidney, testes and brain of normal control compared to negative control group with the heart showing an opposite effect. Treatment with 100 and 200mg/kg naringin slightly increased the GSH levels in the liver to a similar level in normal control. Naringin treatment caused an increase in the brain and testes with no change observed in the GSH levels of the kidney.

Plasma biomarkers of organ damage including creatine kinase (CK), alanine aminotransferase (ALT), Lactate dehydrogenase (LDH), aspartate aminotransferase, alkaline phosphatase and γ -glutamyltransferase and urea were also assayed. There was a significant ($p < 0.05$) increase in aspartate aminotransferase (AST), alkaline phosphatase (ALP), urea and γ -glutamyltransferase (γ -GGT) in diabetic control compared to normal control group. Naringin treatment significantly ($p < 0.05$) reduced the activities of AST, ALP and γ -GGT. Although not significant, increases were observed in lactate dehydrogenase and creatine kinase levels in diabetic control as compared to

normal groups. 50 and 200mg/kg naringin treatment significantly ($p<0.05$) reduced the concentration of plasma urea to a normal level as compared to the diabetic control.

TABLE 2. Effects of naringin on oxidative stress in rat organs

a) Effects of Naringin on Thiobarbituric Acid Reactive Substances (mM/mg protein)

	Liver	Kidney	Testes	Brain	Heart
Diabetic Control	28.23±0.66 ^e	29.43±1.13 ^e	57.15±2.39 ^e	61.51±6.04 ^c	53.80±1.62 ^c
50mg/Kg bw	25.25±0.32 ^d	23.59±0.19 ^d	47.24±0.35 ^d	49.62±0.47 ^b	47.22±0.38 ^{bc}
100mg/Kg bw	22.72±0.26 ^c	22.08±0.22 ^{cd}	43.59±0.46 ^{cd}	46.28±0.50 ^b	42.13±1.34 ^{bc}
200mg/Kg bw	21.51±0.15 ^{bc}	20.16±0.25 ^{bc}	41.09±0.36 ^{bc}	42.96±0.73 ^{ab}	36.42±0.60 ^b
Metformin	20.02±0.26 ^b	18.49±0.18 ^{ab}	38.53±0.54 ^{ab}	39.28±0.32 ^{ab}	32.65±0.67 ^b
Normal Control	16.07±1.00 ^a	17.02±0.27 ^a	35.18±0.85 ^a	35.43±0.73 ^a	17.17±11.18 ^a

b) Effects of Naringin on reduced glutathione (mmol/mg protein)

	Liver	Kidney	Testes	Brain	Heart
Diabetic Control	0.02±0.00 ^a	0.05±0.00 ^a	0.03±0.00 ^a	0.05±0.00 ^a	0.04±0.01 ^a
50mg/Kg bw	0.02±0.00 ^{ab}	0.05±0.00 ^a	0.04±0.00 ^a	0.06±0.01 ^{ab}	0.04±0.01 ^a
100mg/Kg bw	0.03±0.00 ^{bc}	0.05±0.01 ^a	0.04±0.01 ^a	0.06±0.01 ^{ab}	0.03±0.00 ^a
200mg/Kg bw	0.03±0.00 ^{bc}	0.05±0.00 ^a	0.04±0.01 ^a	0.06±0.01 ^{ab}	0.02±0.00 ^a
Metformin	0.03±0.00 ^c	0.05±0.01 ^a	0.05±0.01 ^a	0.06±0.01 ^{ab}	0.03±0.00 ^a
Normal Control	0.03±0.00 ^{bc}	0.08±0.01 ^b	0.07±0.01 ^b	0.09±0.01 ^b	0.03±0.00 ^a

c) Effects of Naringin on glutathione S-transferase (U/mg protein)

	Liver	Kidney	Testes	Brain	Heart
Diabetic Control	0.17±0.01 ^f	0.14±0.01 ^e	0.73±0.05 ^d	0.39±0.01 ^e	0.09±0.00 ^c
50mg/Kg bw	0.15±0.00 ^e	0.1±0.00 ^d	0.56±0.01 ^c	0.3±0.01 ^d	0.06±0.00 ^d
100mg/Kg bw	0.13±0.00 ^d	0.08±0.00 ^c	0.52±0.01 ^{bc}	0.23±0.00 ^c	0.05±0.00 ^c
200mg/Kg bw	0.11±0.00 ^c	0.07±0.00 ^{bc}	0.49±0.01 ^{bc}	0.2±0.00 ^{bc}	0.02±0.00 ^b
Metformin	0.1±0.00 ^b	0.06±0.00 ^b	0.44±0.01 ^b	0.17±0.00 ^b	0.01±0.00 ^a
Normal Control	0.06±0.01 ^a	0.05±0.00 ^a	0.34±0.05 ^a	0.13±0.02 ^a	0.01±0.00 ^a

d) Effects of Naringin on glutathione peroxidase (U/mg protein)

	Liver	Kidney	Testes	Brain	Heart
Diabetic Control	0.21±0.01 ^e	0.22±0.01 ^{bc}	0.37±0.01 ^e	0.42±0.02 ^e	0.52±0.01 ^f
50mg/Kg bw	0.18±0.00 ^d	0.19±0.01 ^{ab}	0.31±0.00 ^d	0.36±0.00 ^d	0.46±0.01 ^c
100mg/Kg bw	0.17±0.00 ^{cd}	0.2±0.02 ^{abc}	0.28±0.00 ^c	0.34±0.00 ^{cd}	0.44±0.00 ^d
200mg/Kg bw	0.16±0.00 ^{bc}	0.16±0.01 ^a	0.26±0.00 ^{bc}	0.32±0.00 ^{bc}	0.42±0.01 ^c
Metformin	0.15±0.00 ^b	0.19±0.01 ^{ab}	0.24±0.00 ^{ab}	0.29±0.00 ^{ab}	0.38±0.00 ^b
Normal Control	0.13±0.00 ^a	0.23±0.01 ^c	0.22±0.00 ^a	0.26±0.00 ^a	0.36±0.01 ^a

e) Effects of Naringin on glutathione reductase (U/mg protein)

	Liver	Kidney	Testes	Brain	Heart
Diabetic Control	0.13±0.01 ^c	0.20±0.01 ^c	0.20±0.04 ^c	0.32±0.03 ^d	0.13±0.01 ^c
50mg/Kg bw	0.10±0.00 ^d	0.16±0.00 ^d	0.11±0.00 ^b	0.21±0.00 ^c	0.10±0.00 ^b
100mg/Kg bw	0.09±0.00 ^{cd}	0.14±0.00 ^{cd}	0.09±0.00 ^{ab}	0.19±0.01 ^{bc}	0.08±0.00 ^{ab}
200mg/Kg bw	0.08±0.00 ^c	0.13±0.00 ^{bc}	0.07±0.00 ^{ab}	0.16±0.00 ^{abc}	0.07±0.00 ^{ab}
Metformin	0.06±0.00 ^b	0.12±0.00 ^b	0.05±0.00 ^{ab}	0.14±0.00 ^{ab}	0.06±0.00 ^b
Normal Control	0.04±0.00 ^a	0.09±0.01 ^a	0.03±0.00 ^a	0.12±0.00 ^a	0.02±0.02 ^a

Values are expressed as mean ± SEM (n=5). Values in the same column for same parameter with different superscripts are significantly different at p<0.05.

TABLE 3. Effects of Naringin on Biochemical organ damage

	Alanine Aminotransferase	Lactate Dehydrogenase (U/L)	γ-glutamyltransferase (U/L)	Creatine Kinase (U/L)	Alkaline Phosphatase (U/L)	Aspartate Aminotransferase (U/L)
Diabetic Control	3.33±1.41 ^a	68.01±9.58 ^a	4.37±0.38 ^c	42.73±3.51 ^a	100.40±17.43 ^b	66.63±10.19 ^c
50mg/Kg bw	6.28±2.19 ^a	67.09±11.55 ^a	2.76±0.00 ^d	42.73±3.50 ^a	77.20±16.89 ^{ab}	27.49±1.00 ^b
100mg/Kg bw	8.73±6.90 ^a	66.32±10.45 ^a	2.30±0.21 ^{cd}	42.73±3.49 ^a	66.32±8.18 ^a	21.14±1.01 ^{ab}
200mg/Kg bw	4.04±1.38 ^a	62.68±14.15 ^a	1.84±0.00 ^b	42.73±3.48 ^a	76.83±13.20 ^{ab}	15.62±0.41 ^{ab}
Metformin	2.21±0.37 ^a	55.88±8.66 ^a	1.47±0.23 ^{ab}	39.06±5.26 ^a	76.46±5.59 ^{ab}	12.31±0.55 ^{ab}
Normal Control	1.69±0.28 ^a	53.30±11.10 ^a	0.92±0.00 ^a	37.91±3.31 ^a	46.26±4.88 ^a	7.35±1.28 ^a

Values are expressed as mean ± SEM (n=5). Values in the same column for same parameter with different superscripts are significantly different at p<0.05.

The effects of naringin on inducible nitric oxide synthase (iNOS) was assessed. The iNOS concentration was significantly (p<0.05) reduced in diabetic control group as compared to normal group. There were significant (p<0.05) increases in the hepatic concentration of iNOS with naringin and metformin treatments with 50 and 200mg/kg giving a similar result to that of normal group.

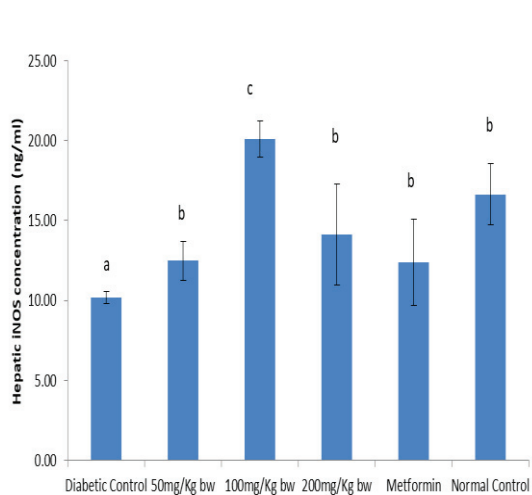


FIGURE 1. Effects of Naringin on iNOS

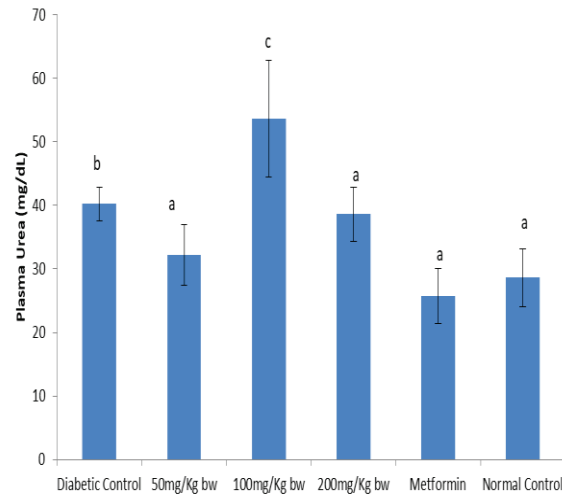


FIGURE 2. Effects of Naringin on urea

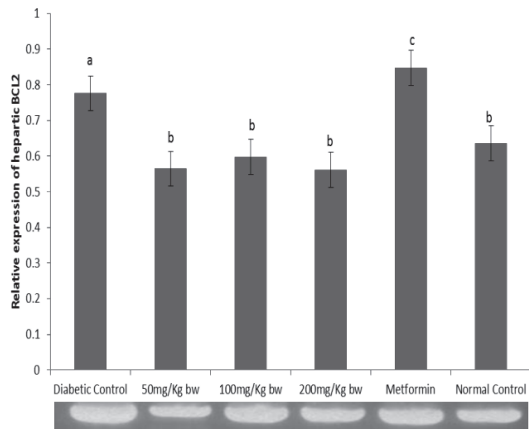


FIGURE 3. Effects of Naringin on BCL-2

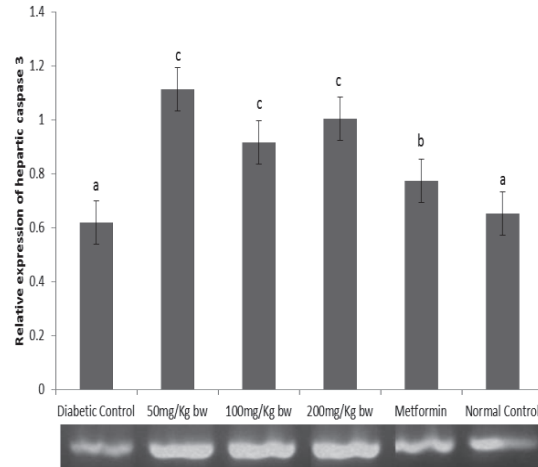


FIGURE 4. Effects of Naringin on Caspase 3

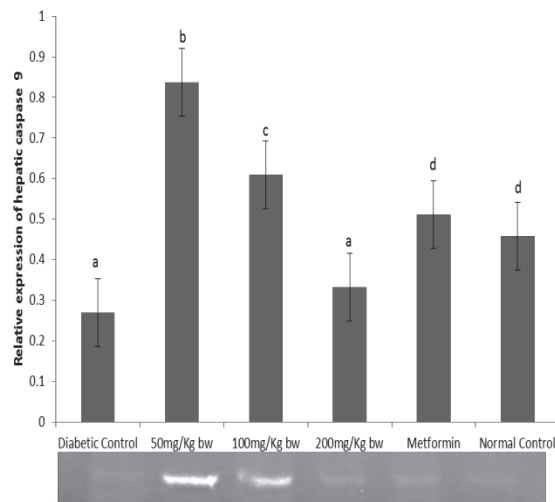


FIGURE 5. Effects of Naringin on Cas9

The relative expression of hepatic BCL-2, caspases 3 and 9 were assessed in relation to apoptosis. There was a significant increase in BCL-2 expression in diabetic control with naringin treatment significantly ($p < 0.05$) reduced the expression to a similar expression level of normal control. Metformin however, significantly ($p < 0.05$) increased BCL-2 expression when compared to diabetic and normal control groups. There was no significant change in the expression of caspase 3 when comparing the diabetic and normal control groups. Treatment with naringin and metformin however, significantly increased the expression of this executor. A significant ($p < 0.05$) reduction of caspase 9 was shown in diabetic control group when compared to normal. 50 and 100mg/kg naringin treatment significantly ($p < 0.05$) increased caspase 9 expression with metformin treatment giving a similar expression to that of the normal control group.

DISCUSSION

In innate immune system, macrophages are vital cells that produce inflammatory cytokine which mop up apoptotic cells. In performing this function, there is an increased production of nitric oxide (NO) which is generally produced by iNOS [17]. iNOS is rarely expressed in cells but plays a significant role in producing NO which carries out its cytotoxic effects on unwanted cells through its affinity for protein-bound iron [18]. From this study, the

reduced plasma concentration of iNOS in diabetic control group when compared with normal and treated group could be attributed to an increased endoplasmic reticulum stress caused by hyperglycemia as reported recently [19]. iNOS has been suggested to play an important role in alleviating plasma ER stress thus, low concentration of iNOS reduces the production of NO in macrophages and a possible increase in ER stress [19]. The reduced concentration of iNOS leading to decreased production of NO could explain the subsequent increase in anti-apoptotic member BCL-2 and inactivation of apoptosis in diabetic control group.

The mechanism of apoptosis in T2DM is not well understood with various results been reported. B-cell apoptosis has been implicated in T2DM in previous studies [6, 21, 22]. Increased expression of anti-apoptotic BCL-2 correlates with the decreased expression of initiator, caspase 9 and effector, caspase 3 when compared to the treated and normal groups which can be best explained as BCL-2 inhibition of apoptosis by inactivating caspases in diabetic rats. This result does not correlate with previous findings of increased apoptosis in T2DM cases [22, 23, 24].

Hyperglycemia in T2DM is known to cause increased production of free radicals which interact with membrane lipids. This result correlates with that of the study with a significant increase in diabetic control TBARS level when compared to normal control. In addition hyperglycemic states cause a rise production of electron donor in form of NADH/H⁺ from important tissues including the muscle and β -cell of pancreas [25]. The electron donor allows single electrons to be transferred to oxygen as against the normal pair of electron producing radicals instead of water [26]. The overproduction of free radicals in T2DM is expected to reduce antioxidant enzymes' activities. Glutathione is regarded as the most important antioxidant system and depletion of the system could cause damage by excessive production of free radicals. This was shown from the study with decreased activities of GRx, GST and GPx in the organs. GPx and GRx play important roles in the glutathione system of antioxidant activities while GPx is also involved in inactivating H₂O₂ [27]. The reduction of these two enzymes correlate with GSH depletion a result that has been implicated in T2DM [28]. Naringin ability to ameliorate oxidative stress in this study seemed to be dose dependent with 200mg/kg bw giving result close to normal control.

Increased liver function enzymes abnormalities are common in cases of T2DM with suggestion that liver function tests could be markers for diabetes mellitus [29, 30]. The significant ($p < 0.05$) increase in plasma ALP and AST of diabetic control compared to normal could be attributed to organ damage thus allowing the release of the enzymes into circulation. However, treatment with naringin showed a significant ($p < 0.05$) reduction with result similar to normal control which could point to the hepatoprotective effect of the flavonoid [31]. However, hepatic cells specific enzyme, ALT showed no significant ($p > 0.05$) change in all treatment groups even though diabetic control group showed an increase compared to normal control. The result is in correlation from a recent study that rated the prevalence of the enzymes' concentration as described by Mathur *et al.* [32] AST prevalence was the highest, followed by that of ALP and ALT. Excessive production of fatty acid in T2DM cases may have a concurrent hepatotoxic effect leading to metabolic irregularities, mitochondria dysfunction amongst others. In addition, increased proinflammatory cytokines and lipid peroxidation may underline the increased production of liver function enzymes [32]. There was a dose dependent decrease in LDH and γ -glutamyltransferase (γ -GGT) levels when treated with naringin from the study. Elevated LDH and γ -GGT in diabetic group can be attributed to organ damage from hyperglycemia and dyslipidemia. This finding is consistent with that of Ishak *et al.* [33].

In addition, kidney diseases have been reported in diabetic patients which is characterised by an increased concentration of urea and creatine kinase [34]. This correlates with the result from this study as in diabetic control group showed increased concentration of urea and creatine kinase (CK). However, naringin treatment showed no significant change in CK concentration but ameliorated the effects of high urea concentration at both 50 and 200mg/kg.

CONCLUSION

This study has been able to add to existing knowledge in terms of the effects of naringin in T2DM. In addition to antioxidant properties, hepatoprotective effects and potential nephroprotective properties were explored. Naringin from the study seem to produce an inverse effect to that of diabetic control thus indicating a role in apoptosis and mechanism of action. More research studies should be carried out to explore the relationship between oxidative stress and apoptosis and possible effects of flavonoids.

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