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NARINGENIN THERAPEUTICS EXERT A PROTECTIVE EFFECT AND ALLEVIATE HYPERGLYCEMIA-INDUCED OXIDATIVE STRESS AND APOPTOSIS IN CARDIAC AND NEURONAL TISSUES OF BALB/C DIABETIC MICE

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Keywords:

Naringenin, Streptozotocin, Hyperglycemia, Oxidative stress, Apoptosis

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ABSTRACT: The continuous search to search for a naturally occurring medicinal compound that could be a suitable adjunct to existing therapies for treating one of the growing global health emergencies in diabetes is a never-ending one. Naringenin [2, 3-dihydro-5, 7-dihydroxy- 2 -(4-hydroxyphenyl) - 4H-1-benzopyran-4-one], a naturally occurring flavonoid abundantly present in grapes, grapefruit, and citrus fruits have been found to confer different pharmacological activities. Thus, keeping its potential medicinal properties in mind, our aim of the study was an in-depth study of intraperitoneal treatment of naringenin, especially at the levels of oxidants, lipidemic activities, and anti-apoptotic activity in tissues such as the heart and brain, so that it could be better evaluated as a potential therapeutic agent. Our study demonstrates that naringenin treatment reduced hyperglycemia-inducedoxidative stress by activating various intracellular anti-oxidant enzymes and preventing apoptosis by initiating the translocation of Bcl-2 to the mitochondria, partially affecting the release of mitochondrial cytochrome c to the cytoplasm and preventing the activation of caspase-3 and caspase-9, thus preventing cell death. The transmission electron microscopic (TEM) studies confirm the protective effect of naringenin in tissue ultrastructure caused by oxidative stress. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay confirms that fewer apoptotic cells were present in naringenin-treated diabetic mice. Also, naringenin treatment in diabetic mice has a significant protective effect on lipid abnormalities caused by hyperglycemia. Therefore, naringenin could be beneficial in ameliorating some aspects of diabetic complications by protecting the heart and brain tissues of diabetic mice through its antioxidant and anti-apoptotic effects.

INTRODUCTION: Diabetes is one of the global health emergencies of the 21st century as stated by International Diabetes Federation [1].

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Although insulin's availability in 1921 improved people's life expectancy drastically; however, it also became clear that long-term living with diabetes exposed the body to many complications leading to morbidity and mortality from both type 1 and type 2 diabetes. The importance of protecting the body from hyperglycemia cannot be overstated, and its detrimental effects can be separated into macrovascular and microvascular complications. Many diabetic complications are caused by

oxidative stress and excessive free radical creation, and prolonged hyperglycemia is a significant inducer of free radical production [2]. Numerous studies have concluded a correlation between diabetes and oxidative stress, with oxidative stress being one of the significant contributors to the development and progression of diabetes [3]. In contrast, increased ROS may cause more severe and irreversible cell damage by oxidation of DNA, RNA, carbohydrates, proteins, and lipids, ultimately leading to physiological dysfunction and cell death through apoptosis or necrosis [4].

Herbal remedies are increasingly becoming more beneficial in managing diabetes and have been used since immemorial. Over many decades, experimental evidence supporting the concept that flavonoids with strong antioxidant activities ameliorating oxidative stress and prevent damage to various organs has emerged [5, 6, 7]. Flavonoids may exert their protective role through several mechanisms, including quenching secondary lipid oxidation products, chelating metals, inactivating peroxides, scavenging free radicals [8], galvanizing and defending intercellular antioxidant enzymes, or reacting with ROS through hydrogen atom transfer, single electron transfer or metal chelation [9].

One such naturally occurring flavonoid compound is Naringenin (2, 3-dihydro-5, 7-dihydroxy-2-(4-hydroxyphenyl) - 4H - 1-benzopyran – 4 - one) abundantly present in grapes, grapefruit, cooked tomato paste, and citrus fruits [10]. It has a molecular weight of 272.26 ($C_{15}H_{12}O_5$) and exists predominantly in nature in two forms: the glycosylated form (naringin or naringenin-7-O-glucoside) and the aglycosylated form (naringenin) [11]. Naringin can then be hydrolyzed into naringenin by the liver enzyme naringinase.

Naringenin has been found to confer different pharmacological activities, including antioxidant behaviour [12], inhibition of vascular smooth muscle proliferation [13], anti-viral and anti-inflammatory [14], antibacterial activity [15, 16], provides protections against various disorder such as cardiovascular disease [17], neurodegenerative diseases [18], malignant tumors [19], inhibiting the progression of fibrosis in various organs [20]. With such a promising and comprehensive pharmacological advantage, an in-depth study of the intraperitoneal treatment of naringenin, especially the regulatory or deregulatory effects on oxidants, hyperlipidemia, apoptosis-related proteins, and cell death in diabetic heart and brain tissues is required to understand the state entirely. Thus, the goal of our research study was to find out more about the action of naringenin so that it could be better evaluated as a potential therapeutic agent.

MATERIALS AND METHODS:

Chemicals: All chemicals used were of analytical grade and were used without further purification. (±) Naringenin (95%), sodium dodecyl sulphate (SDS), bovine serum albumin (BSA), streptozotocin (STZ), metformin, 1-chloro-2,4, dinitro benzene (CDNB), pyrogallol, 1,1,3,3-tetraethoxypropane (TEP), thiobarbituric acid (TBA), diethylenetriaminepenta-acetic acid (DETAPAC), 3, 3’, 5, 5’-Tetramethylbenzidine (TMB), and 4',6-diamidino-2-phenylindole (DAPI) were procured from Sigma-Aldrich Co. USA. *In situ* cell death detection kit, fluorescein for Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was obtained from Roche, Switzerland. Primary antibodies like an anti-Bcl-2 antibody, anti-caspase 3 antibody, anti-caspase9, and anti-β-actin antibodies were procured from Merck life science Pvt. Ltd. India, Sigma-Aldrich Co. USA, and Abcam, UK, respectively. All other chemicals used were procured from Sisco Research Laboratory (SRL), Hi-Media, and SD Fine-Chem Ltd., India.

Experimental Animals: In this study, male Swiss albino mice (Balb/C strain) weighing 25-30 g were employed. Mice were kept in clean polypropylene cages with stainless steel grill tops under temperature control (25±2°C) with a 12-hour day-night cycle in the animal rooms. They were given a conventional laboratory meal from Amrut Laboratory in Pune, India, and unlimited water. All animal work, including animal care, studies, and procedures, was done following the Institutional Ethics Committee (Animal models), North-eastern Hill University, Shillong, India.

Induction of Diabetes: Prior to injections, mice were housed in a separate cage and fasted for 6 hours. Diabetes was then induced with a single high dose of intraperitoneal injection of streptozotocin [150mg/kg body weight (b.w.)]

dissolved in 0.1M cold citrate buffer (pH 4.5), followed by another intraperitoneal injection of 1ml of 5% glucose solution to counter the hypoglycemic shock [21]. After 72 hours, fasting blood glucose levels were measured using an SD Check glucometer (SD Biosensor Inc., Korea), and animals with blood glucose concentrations of 200mg/dl or above were chosen.

Study of the Effects of Naringenin on Lipid Profile:

Experimental Design: Four different groups of mice were selected, and doses were injected every alternate day for 28 days intraperitoneally.

Group A: Control mice administered with citrate buffer, pH 4.5 (NU).

Group B: Diabetic untreated mice (STZ induced) with citrate buffer as the vehicle (DU).

Group C: Diabetic mice administered with 50mg/kg b.w. naringenin dissolved in 0.5% carboxymethyl cellulose (DNT).

Group D: Diabetic mice administered with 50mg/kg b.w. metformin (DMT). At the end of the treatment, the mice were sacrificed, and the serum was collected by retro-orbital puncture and subsequently performed for the lipid profile study using coral kits.

Study of the Effects of Naringenin on Oxidative Stress

Experimental Design: For all the *in-vivo* experiments, mice were divided into the following groups, each comprising six mice.

Group A: Normal untreated mice which received only citrate buffer (10 ml/kg b.w. with pH 4.5) as a vehicle (NU).

Group B: Diabetic untreated mice induced by streptozotocin injections intraperitoneally with citrate buffer as the vehicle (DU)

Group C: Diabetic mice treated with naringenin (50mg/kg b.w.) dissolved in 0.5% carboxymethyl cellulose by intraperitoneal injection every alternate day for 28 days (DNT).

Group D: Diabetic mice treated with L-ascorbic acid (50mg/kg b.w.) by intraperitoneal injection every alternate day for 28 days (DVT). At the end of the experimental period, the mice were fasted for 6hr and anesthetized before sacrificing. Heart and brain tissues were excised and washed with homogenization buffer (pH 7.4) to remove excess blood and debris, maintain the tissues' integrity, and store at -80°C until further analysis.

Antioxidative Enzyme Activity Assay: The activity of Mn-SOD and Cu/Zn-SOD was assayed by the method of Marklund *et al.* [23]. The method of Aebiwas employed to assay the activity of CAT [24]. The activity of SODs and CAT are expressed as units/milligram protein/min. The activity of GPx was determined by the method of Rotruk [25] and expressed as micromoles of reduced glutathione consumed/min/mg protein. The activity of GST was assayed by Habig *et al.* [26] and expressed as micromoles of 1-chloro, 2-4 dinitrobenzene formed/min/mg protein.

Lipid Peroxidation Assay: MDA, a marker of lipid peroxidation, was measured as thiobarbituric acid-reacting substances (TBARS) as per the method of Ohkawa *et al*. and expressed as nmol/mg protein [27].

Histo-pathological Studies: The mice tissues *i.e.,* heart and brain tissues of normal untreated, diabetic untreated, diabetic treated with naringenin and ascorbic acid, were processed according to the method of Kiernan with some modifications [28]. Tissue sections were stained with hematoxylin and eosin (H&E) and examined under a compound microscope.

Ultra-morphological Studies under Transmission Electron Microscope (TEM): For TEM studies, various sections of heart and brain tissues of experimental groups were prepared following the method described by Hayat with some modifications [29] and viewed under TEM (JEM-100 CX II)

Study of the Effects of Naringenin on Apoptosis:

Experimental Design: The experimental design is the same as in section 2.5.

Effect of Naringenin on Apoptosis-associated Proteins Expression: Western Blot analysis:

Tissue Homogenization: Heart and brain tissues were extracted in 10% (w/v) extraction buffer

containing 10 mM HEPES (pH 7.4), 0.2 M mannitol, 50 mM sucrose, and 1 mM EDTA. Tissue homogenate was centrifuged at 13000g for 15 minutes at 40C, according to Kaushal *et al* [30]. The supernatant was immediately collected and stored at -80°C for protein expression analysis.

Western Blot Analysis: Western blot analysis was performed using anti-caspase-3 (1:1000), anti-caspase-9 (1:1000), anti-Bcl-2 (1:500), and anti-β-actin (1:1000) primary antibodies following our previous method as mentioned in Patar *et al* [22].

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP Nick-end Labeling (TUNEL) Assay: 'In situ cell death detection kit, fluorescein' kit was used for TUNEL assay following the protocol in the kit obtained fromRoche, Switzerland.

Statistical Analysis: The statistical tool "IBM SPSS Statistics 19.0 for Windows" was used to perform one-way analysis of variance (ANOVA) followed by Tukey's post hoc test to examine differences between experimental groups. The data were presented as mean ± SEM. The statistical significance level was chosen at p <0.05, P<0.01 and P<0.001.

RESULTS:

Anti-hyperlipidemia Studies: Fig. 1 depicts the mean levels of lipid profile markers in each group. Diabetic untreated mice showed significantly (P < 0.001) higher levels in all parameters, including total cholesterol (TC), triglycerides (TG), and low-density lipoprotein-cholesterol (LDL-C), except high-density lipoprotein-cholesterol (HDL-C), which had significantly (P < 0.001) lower levels.

Interestingly, as compared to diabetic untreated mice, naringenin and metformin-treated diabetic mice had considerably (P < 0.001) reduced mean levels of TC, TG, and LDL-C, as well as a considerable (P < 0.001) rise in HDL-C. There were no significant differences in the data obtained in naringenin-treated (Group C) mice vs metformin-treated (Group D) animals.

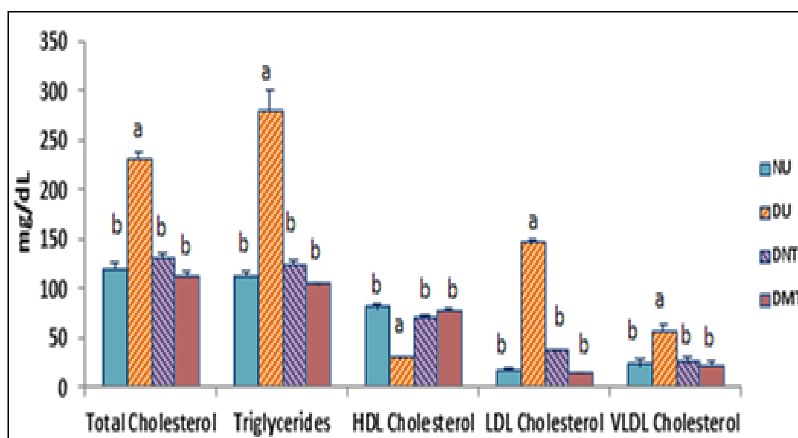

FIG. 1: MEAN LEVELS OF THE LIPID PROFILE MARKERS IN SWISS ALBINO MICE EXPRESSED IN MG/DL AND REPRESENT THE MEAN ± SEM OF OBSERVATIONS MADE ON SIX MICE IN EACH GROUP. [a]Statistically significant difference (*P* < 0.001) when compared with NU values. [b]Statistically significant difference (*P* < 0.001) when compared with DU values.

In-vivo Antioxidant Studies:

Antioxidative Enzyme Activities in the Heart: Table 1 displays the mean activity of SOD, CAT, GPx, and GST in mice heart tissue.

The mean activities of these enzymatic antioxidants in the cardiac tissue of diabetic untreated (Group B) mice were considerably (P <0.001) lower than those in normal untreated (Group A) mice. In diabetic mice treated with naringenin (Group C) and vitamin C (Group D), mean enzyme activities were considerably (P <0.001) greater than in diabetic untreated (Group B) mice.

Surprisingly, no significant difference was seen between naringenin-treated (Group C) and vitamin C-treated (Group D) mice. However, the mean values of all the enzymes in diabetic untreated mice were significantly lower than those of untreated normal mice, naringenin, and vitamin C treated mice.

TABLE 1: MEAN ACTIVITIES OF SOD (UNITS/MILLIGRAM PROTEIN/MIN), CAT (UNITS/MILLIGRAM PROTEIN/MIN), GPX (MICROMOLES OF REDUCED GLUTATHIONE CONSUMED/MIN/MG PROTEIN) AND GST (MICROMOLES OF 1-CHLORO,2-4 DINITROBENZENE FORMED/MIN/MG PROTEIN) IN HEART OF EXPERIMENTAL GROUPS. VALUES REPRESENT THE MEAN ± SEM FOR THE OBSERVATIONS MADE ON SIX MICE IN EACH GROUP

Groups	Superoxide dismutase		Catalase	Glutathione Peroxidase	Glutathione-s-transferase
	Cu/Zn-SOD	Mn-SOD			
Group A (Normal Untreated)	0.0060 ± 0.002^{b}	0.062 ± 0.002^{b}	0.070 ± 0.002^{b}	3.378 ± 0.417^{b}	0.050 ± 0.005^{b}
Group B (Diabetic Untreated)	0.0336 ± 0.003^{b}	0.030 ± 0.003^{b}	0.037 ± 0.001^{a}	0.711 ± 0.200^{a}	0.025 ± 0.003^{a}
Group C (Diabetic + Naringenin Treated)	0.052 ± 0.004^{b}	0.053 ± 0.004^{b}	$0.056 \pm 0.002^{a,b}$	2.544 ± 0.414^{b}	0.041 ± 0.002^{b}
Group D (Diabetic + Vitamin C-Treated)	0.054 ± 0.002^{b}	0.056 ± 0.002^{b}	0.061 ± 0.001^{b}	2.711 ± 0.319^{b}	0.043 ± 0.001^{b}

[a] Statistically significant difference ($P < 0.001$) when compared with Group A values. [b] Statistically significant difference ($P < 0.001$) when compared with Group B values.

Antioxidative Enzyme Activities in the Brain: Table 2 depicts the mean activities of SOD, CAT, GPx, and GST in brain tissue. Values showed significantly ($P < 0.001$) lower mean activities of these enzymatic antioxidants in the brain tissue of diabetic untreated (Group B) mice than those in normal untreated (Group A) mice. Conversely, significantly ($P< 0.001$) higher mean activities of these enzymes were observed in diabetic mice treated with naringenin (Group C) and vitamin C (Group D) than those in diabetic untreated (Group B) mice. Remarkably, there was no significant difference between naringenin-treated (Group C) and vitamin C-treated (Group D) mice. However, the mean values of all the enzymes in diabetic untreated mice were significantly lower than in other experimental groups.

TABLE 2: MEAN ACTIVITIES OF SOD (UNITS/MILLIGRAM PROTEIN/MIN), CAT (UNITS/MILLIGRAM PROTEIN/MIN), GPX (MICROMOLES OF REDUCED GLUTATHIONE CONSUMED/MIN/MG PROTEIN) AND GST (MICROMOLES OF 1-CHLORO,2-4 DINITROBENZENE FORMED/MIN/MG PROTEIN)IN THE BRAIN OF EXPERIMENTAL GROUPS. VALUES REPRESENT THE MEAN ± SEM FOR THE OBSERVATIONS MADE ON SIX MICE IN EACH GROUP

Groups	Superoxide dismutase		Catalase	Glutathione Peroxidase	Glutathione-s-transferase
	Cu/Zn-SOD	Mn-SOD			
Group A (Normal Untreated)	0.074 ± 0.002^{b}	0.085 ± 0.003^{b}	0.093 ± 0.004^{b}	4.089 ± 0.279^{b}	0.068 ± 0.007^{b}
Group B (Diabetic Untreated)	0.027 ± 0.002^{b}	0.035 ± 0.002^{b}	0.048 ± 0.001^{a}	1.089 ± 0.079^{a}	0.032 ± 0.002^{a}
Group C (Diabetic + Naringenin Treated)	0.066 ± 0.004^{b}	0.072 ± 0.001^{b}	$0.068 \pm 0.002^{a,b}$	3.589 ± 0.288^{b}	0.059 ± 0.005^{b}
Group D (Diabetic + Vitamin C-Treated)	0.069 ± 0.003^{b}	0.075 ± 0.004^{b}	0.071 ± 0.001^{b}	3.889 ± 0.321^{b}	0.062 ± 0.005^{b}

[a] Statistically significant difference ($P < 0.01$) when compared with Group A values. [b] Statistically significant difference ($P < 0.01$) when compared with Group B values.

Lipid Peroxidation Studiesin the Heart and Brain Tissue: The mean levels of malondialdehyde (MDA) in the heart and brain tissues of diabetic untreated (Group B) mice was significantly ($P< 0.001$) higher than in normal untreated (Group A) mice. As shown in **Table 9**, the percentage increase from normal untreated mice to diabetic mice was found out to be 75% and 68% in the brain and heart, respectively. Remarkably, while the mean levels of MDA in diabetic mice treated with naringenin (Group C) and in those treated with vitamin C (Group D) were significantly ($P< 0.001$) lower than in diabetic untreated (Group B) mice, whereas these levels were not significantly

different to those in normal untreated (Group A) mice. The percentage reduction from diabetic mice in naringenin-treated mice was found to be 37% and 36%, and in vitamin C-treated mice, 41% and 39% in the brain and heart, respectively. There is no significant difference between the mean values in Group C and Group D mice **Fig. 2**.

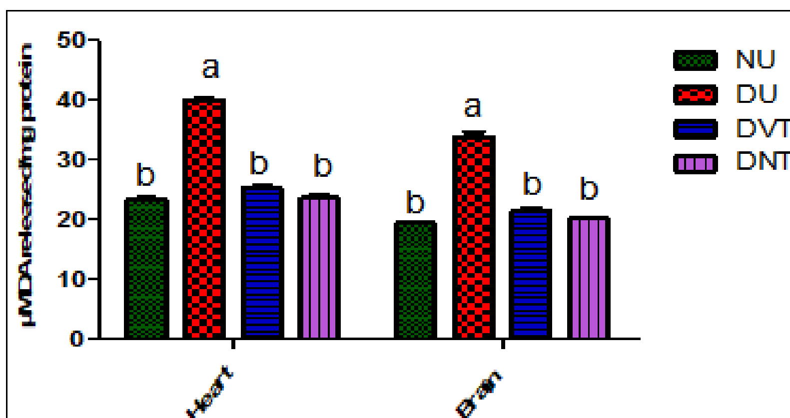

FIG. 2: LIPID PEROXIDATION STUDIES. [a] Statistically significant difference (*P* < 0.01) when compared with Group A values. [b] Statistically significant difference (*P* < 0.01) when compared with Group B values.

Histopathological Studies:

Histopathological Examination of Heart Tissue: **Fig. 3A** depicts the histoarchitecture of the cardiac tissue in all four groups.

Heart tissue from normal untreated (NU) mice showed typical architecture of heart cells with intact interstitium and myofibers and normal cardiomyocytes. Disarray and collapse of myofibers and cardiac degeneration involving enhanced cytoplasmic vacuolization, fiber thinning, and interstitium widening were found in diabetic untreated (DU) mice.

In naringenin-treated (DNT) and vitamin C-treated (DVT) animals, restoration of near normal architecture with a near normal cardiomyocytes complete frame was found with very little vacuolization.

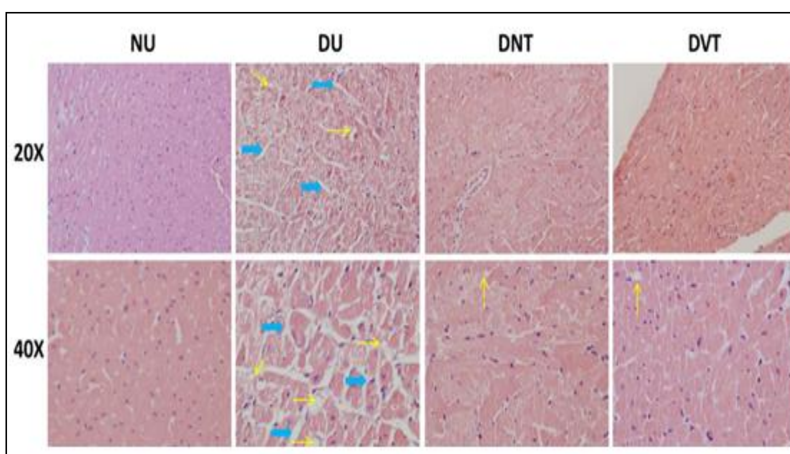

FIG. 3A: HISTOARCHITECTURE OF HEART OF SWISS ALBINO MICE. HEMATOXYLIN-EOSIN STAINING WITH 20X AND 40XMAGNIFICATION

Histopathological Examination of Brain Tissue: The histoarchitecture of the brain tissue in all four groups is shown in **Fig. 3B**.

Normal brain walls and normal glial cells with intact frameworks were noted in the brain tissue of normal untreated (NU) mice. Wall collapse, increased proliferation of the glial cells, and frame loss were observed in diabetic untreated (DU) mice. Interestingly, restoration of near-normal architecture with normal glial cells and a near-intact frame was observed in both naringenin-treated (DNT) and vitamin C-treated (DVT) mice.

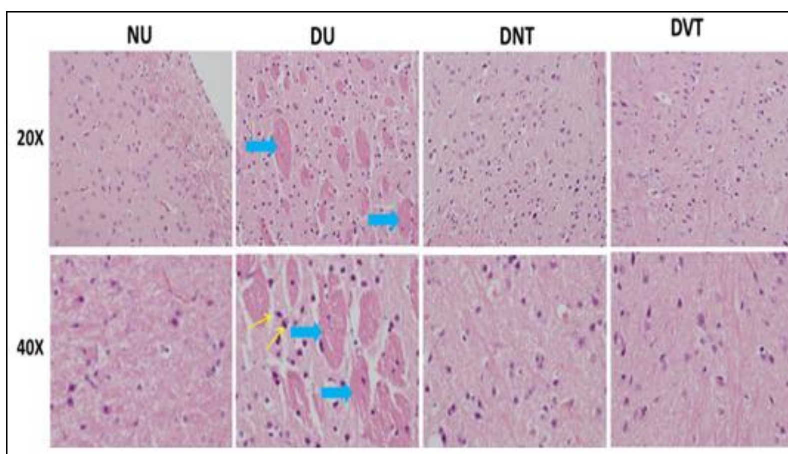

FIG. 3B: HISTOARCHITECTURE OF BRAIN OF SWISS ALBINO MICE. HEMATOXYLIN-EOSIN STAINING WITH 20X AND 40 X MAGNIFICATIONS

Transmission Electron Micrographs:

Transmission Electron Micrographs of Heart Tissue: The electron micrographs of the cardiac tissues of all four groups are shown in **Fig. 4A**. Mitochondria NU appeared relatively well-integrated, elongated, and organized along the interfibrillar bands of the cell. Also, highly packed symmetric myofibrils in the Z-lines were observed. Contrarily, DU shows disorganized and degenerated myofibrils and altered Z-lines. However, the DNT and DVT-treated tissues show symmetrical and well-organized myofibrils and intact Z-lines with well-integrated mitochondria. Some mitochondria (*), although, shows damage to the inner membrane.

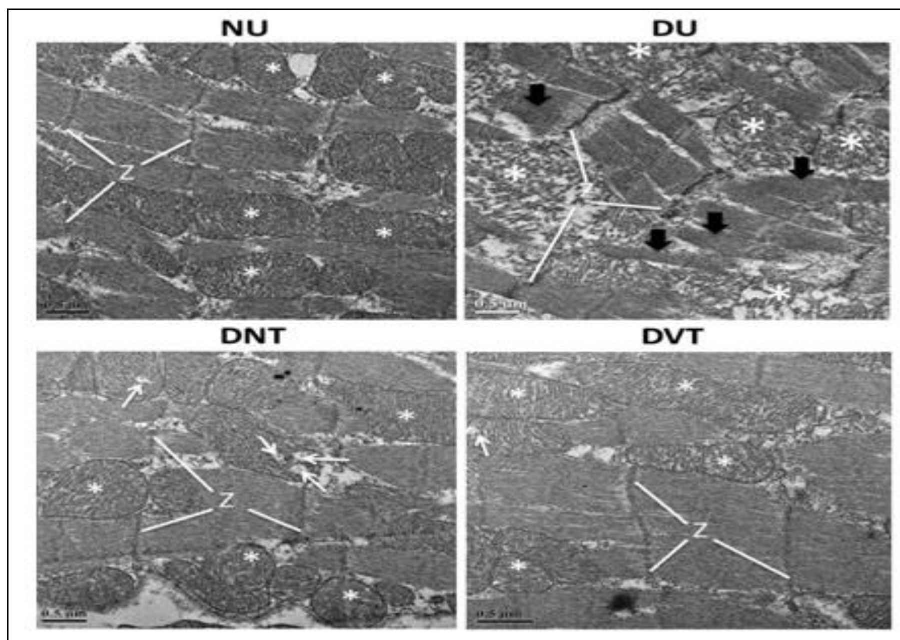

FIG. 4A: REPRESENTATIVE PHOTOGRAPH OF ELECTRON MICROSCOPY SHOWING THE CHANGES IN CARDIAC TISSUE IN A SECTION OF THE HEART IN SWISS ALBINO MICE. MAGNIFICATION: 5, 000X

Transmission Electron Micrographs of Brain Tissue: The electron micrographs of the brain tissues of all four groups are shown in **Fig. 4B**.

Normal mice show compact and arranged myelinated axons with intact mitochondria. In contrast, diabetic mice showed large, irregularly shaped collapsing myelinated fiber with a loose myelin sheath and a small thin myelin sheath.

On the other hand, DNT and DVT showed improved myelin sheath suggesting the potential of treatment to hinder the nerve damage of diabetic animals partially.

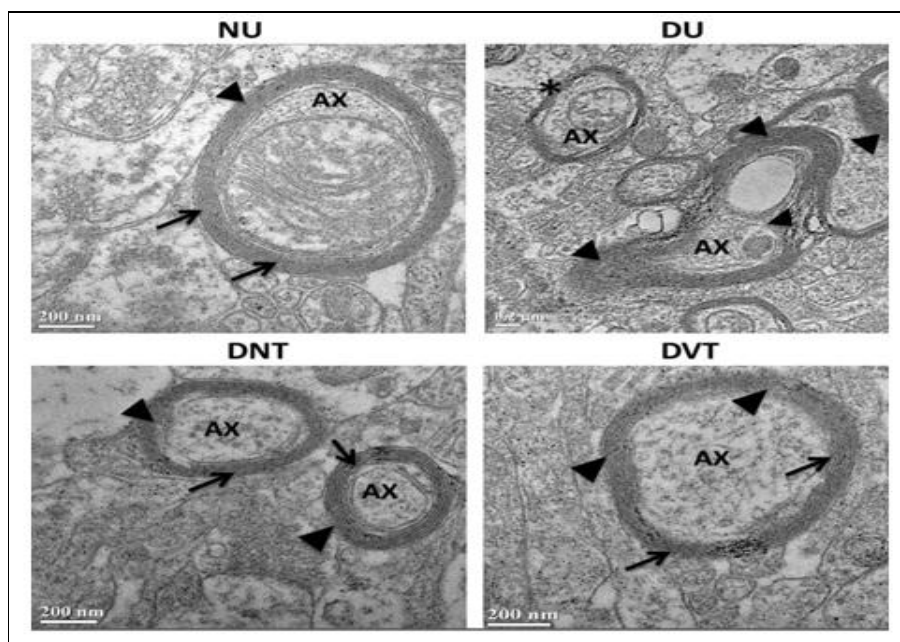

FIG. 4B: REPRESENTATIVE PHOTOGRAPH OF ELECTRON MICROSCOPY SHOWING THE CHANGES IN NEURONAL CELLS IN A SECTION OF BRAIN TISSUE IN SWISS ALBINO MICE. MAGNIFICATION: 10,000X

Western Blot Analysis:

Effect of naringenin on Caspase-3 expression: In the heart, caspase-3 activity in the STZ-induced DU group was substantially greater than in the NU group (1.51± 0.06 vs 0.53 ± 0.01). After 28 days, both vitamin C and naringenin treatment significantly reduced caspase-3 activity (0.56 ± 0.04 and 0.77± 0.07) compared to diabetic untreated mice. When compared to the NU mouse group, hyperglycemia increased 184% heart tissue caspase-3 activity in DU mice, while therapy lowered by 48% and 62% in DNT and DVT animals, respectively **Fig. 5A.**

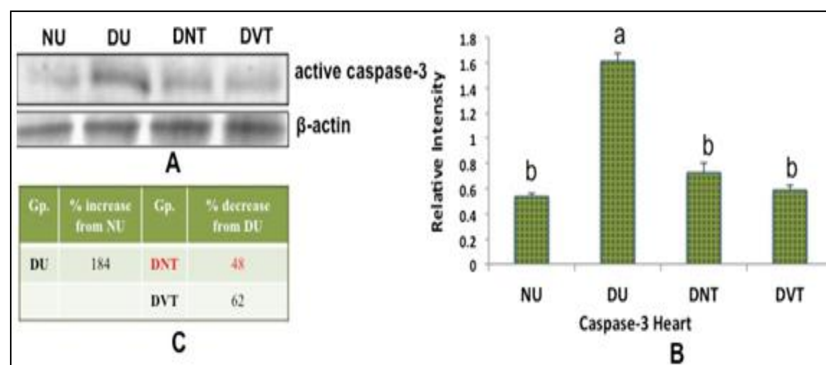

FIG. 5A: A WESTERN BLOT ANALYSIS SHOWING Β-ACTIN AND CASPASE-3 EXPRESSION IN HEART TISSUE OF NU, DU, DNT AND DVT. QUANTITATIVE DENSITOMETRY IS EXPRESSED AS THE RATIO BETWEEN CASPASE-3 AND THE COMPARATIVE PROTEIN Β-ACTIN. (MEAN ± SEM), N = 6. B) RELATIVE INTENSITY OF CASPASE-3 EXPRESSION AFTER NORMALIZING WITH Β-ACTIN IN HEART TISSUE OF EXPERIMENTAL GROUPS. C) PERCENTAGE INCREASE AND DECREASE IN CASPASE-3 ACTIVITY WAS CALCULATED BY COMPARING DU WITH NU, WHILE DVT AND DNT WITH DU MICE. [a] Statistically significant difference ($P < 0.001$) when compared with NU values. [b] Statistically significant difference ($P < 0.001$) when compared with DU values.

In the brain, caspase-3 activity in the STZ-induced DU group was substantially greater than in the NU group (1.21 ± 0.02 vs 0.23 ± 0.01). After 28 days, both vitamin C and naringenin significantly reduced caspase-3 activity (0.28 ± 0.03 and 0.32 ± 0.03) compared to diabetic untreated mice. When compared to the NU mouse group, hyperglycemia increased brain tissue caspase-3 activity by 427% in DU mice. At the same time, therapy lowered it by 73% and 76% in DNT and DVT animals, respectively **Fig. 5B.**

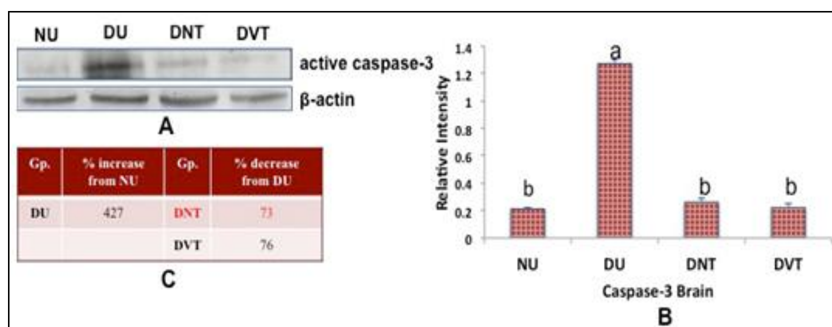

FIG. 5B: A WESTERN BLOT ANALYSIS SHOWING Β-ACTIN AND CASPASE-3 EXPRESSION IN BRAIN TISSUE OF NU, DU, DNT AND DVT. QUANTITATIVE DENSITOMETRY IS EXPRESSED AS THE RATIO BETWEEN CASPASE-3 AND THE COMPARATIVE PROTEIN Β-ACTIN. (MEAN ± SEM), N = 6. B) CASPASE-3 ACTIVITY IN BRAIN TISSUE OF NU, DU, DVT, AND DNT MICE AFTER 28-DAY TREATMENT. C) PERCENTAGE INCREASE AND DECREASE IN CASPASE-3 ACTIVITY WERE CALCULATED BY COMPARING DU WITH NU, WHILE DVT AND DNT WITH DU MICE. [a] Statistically significant difference (P< 0.001) when compared with NU values. [b] Statistically significant difference (P< 0.001) when compared with DU values.

Effect of Naringenin on Caspase-9 Expression: In the heart, caspase-9 activity in the STZ-induced DU group was substantially greater than in the NU group (1.11 ± 0.02 vs 0.35 ± 0.01). After 28 days, both vitamin C and naringenin significantly reduced caspase-9 activity (0.41 ± 0.02 and 0.43 ± 0.01) compared to diabetic untreated mice. When compared to the NU mouse group, hyperglycemia increased heart tissue caspase-9 activity by 212% in DU mice, while therapy lowered it by 61% and 63 % in DNT and DVT mice, respectively **Fig. 6A.**

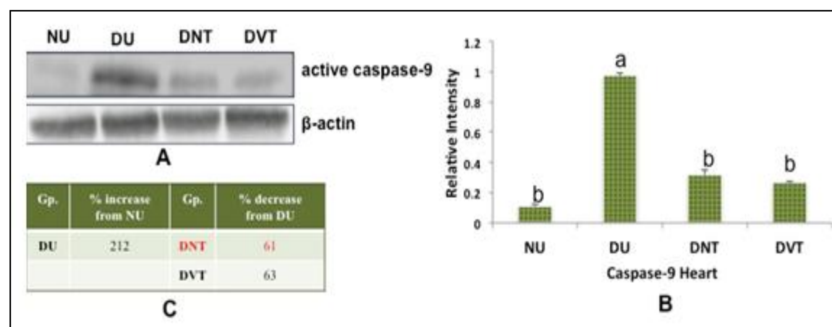

FIG. 6A: A WESTERN BLOT ANALYSIS SHOWING Β-ACTIN AND CASPASE-9 EXPRESSION IN HEART TISSUE OFNU, DU, DNTANDDVT. QUANTITATIVE DENSITOMETRY IS EXPRESSED AS THE RATIO BETWEEN CASPASE-9 AND THE COMPARATIVE PROTEIN Β-ACTIN. (MEAN ± SEM), N = 6. B) CASPASE-9 ACTIVITY IN HEART TISSUE OF NU, DU, AND DVT, AND DNT MICE AFTER 28-DAY TREATMENT. C) PERCENTAGE INCREASE AND DECREASE IN CASPASE-9 ACTIVITY WERE CALCULATED BY COMPARING DU WITH NU, WHILE DVT AND DNT WITH DU MICE. [a] Statistically significant difference (*P*< 0.001) when compared with NU values. [b] Statistically significant difference (*P*< 0.001) when compared with DU values.

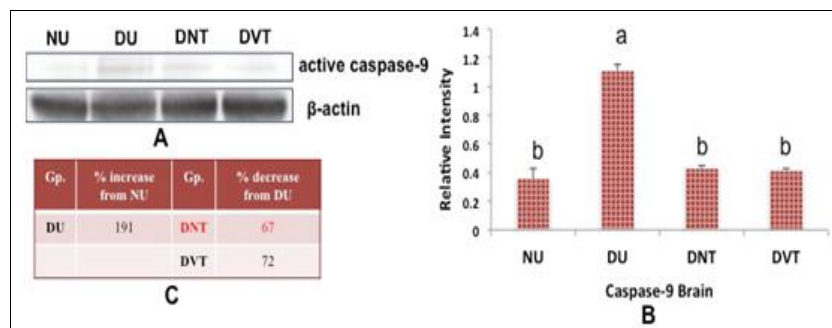

FIG. 6B: WESTERN BLOT ANALYSIS SHOWING Β-ACTIN AND CASPASE-9 EXPRESSION IN BRAIN TISSUE OFNU, DU, DNT, ANDDVT. QUANTITATIVE DENSITOMETRY IS EXPRESSED AS THE RATIO BETWEEN CASPASE-9 AND THE COMPARATIVE PROTEIN Β-ACTIN. (MEAN ± SEM), N = 6. B) CASPASE-9 ACTIVITY IN BRAIN TISSUE OF NU, DU, DVT, AND DNT MICE AFTER 28-DAY TREATMENT. C) PERCENTAGE INCREASE AND DECREASE IN CASPASE-9 ACTIVITY WERE CALCULATED BY COMPARING DU WITH NU, WHILE DVT AND DNT WITH DU MICE. [a] Statistically significant difference (P< 0.001) when compared with NU values. [b] Statistically significant difference (P< 0.001) when compared with DU values.

In the brain, caspase-9 activity in the STZ-induced DU group was substantially greater than in the NU group (0.97 ± 0.01 vs 0.1 ± 0.01). After 28 days, both vitamin C and naringenin significantly reduced caspase-9 activity (0.26 ± 0.02 and 0.31 ± 0.01) compared to diabetic untreated mice. When compared to the NU mouse group, hyperglycemia increased by 191% brain tissue caspase-9 activity in DU mice, while therapy lowered by 67% and 72% in DNT and DVT animals, respectively **Fig. 6B.**

Effect of Naringenin on Bcl-2 Expression: In the heart, Bcl-2 activity in the STZ-induced DU group was substantially lower than in the NU group (0.28±0.02 versus 0.82± 0.01).

After 28 days of therapy, both vitamin C and naringenin significantly increased Bcl-2 activity (0.52± 0.02 and 0.56± 0.01) compared to diabetic untreated mice.

Hyperglycemia was observed to reduce 65% of heart tissue Bcl-2 activity in DU mice when compared to the NU mice group. At the same time, it is increased by 95% and 82% in DNT and DVT animals, respectively when compared to DU mice **Fig. 7A.**

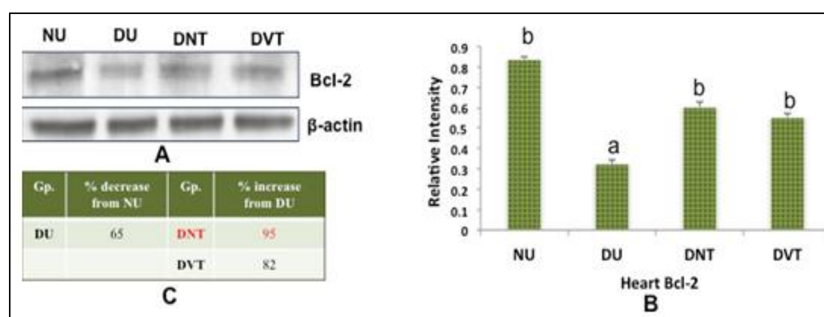

FIG. 7A: A WESTERN BLOT ANALYSIS SHOWING Β-ACTIN AND BCL-2 EXPRESSION IN HEART TISSUE OFNU, DU, DNTANDDVT. QUANTITATIVE DENSITOMETRY IS EXPRESSED AS THE RATIO BETWEEN BCL-2 AND THE COMPARATIVE PROTEIN Β-ACTIN. (MEAN ± SEM), N = 6. B) BCL-2 ACTIVITY IN HEART TISSUE OF NU, DU, DVT, AND DNT MICE AFTER 28-DAY TREATMENT. C) PERCENTAGE INCREASE AND DECREASE IN BCL-2 ACTIVITY WERE CALCULATED BY COMPARING DU WITH NU AND DVT AND DNT WITH DU MICE. [a] Statistically significant difference (*P*< 0.001) when compared with NU values. [b] Statistically significant difference (*P*< 0.001) when compared with DU values.

In the brain, Bcl-2 activity in the STZ-induced DU group was considerably lower than in the NU group (0.17± 0.02 vs 0.63± 0.02). After 28 days of therapy, both vitamin C and naringenin significantly increased Bcl-2 activity (0.46 ±0.02 and 0.42± 0.01) compared to diabetic untreated mice. Hyperglycemia was observed to reduce 72% of brain tissue Bcl-2 activity in DU mice when compared to the NU mice group, while it raised 139% and 161% in DNT and DVT mice respectively when compared to DU mice **Fig. 7B.**

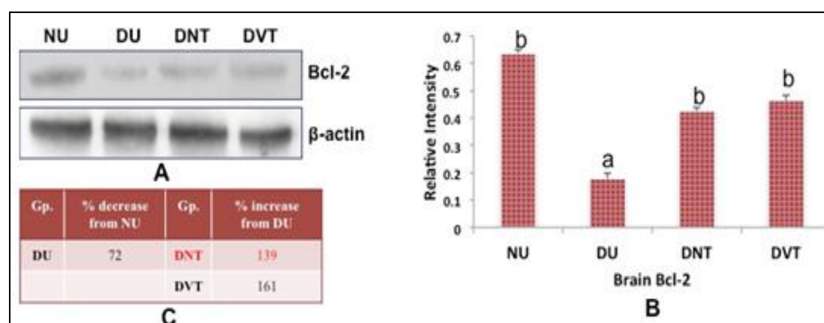

FIG. 7B: A WESTERN BLOT ANALYSIS SHOWING Β-ACTIN AND BCL-2 EXPRESSION IN BRAIN TISSUE OFNU, DU, DNTANDDVT. QUANTITATIVE DENSITOMETRY IS EXPRESSED AS THE RATIO BETWEEN BCL-2 AND THE COMPARATIVE PROTEIN Β-ACTIN. (MEAN ± SEM), N = 6. B) BCL-2 ACTIVITY IN THE BRAIN TISSUE OF NU, DU, DVT, AND DNT MICE AFTER 28-DAY TREATMENT. C) PERCENTAGE INCREASE AND DECREASE IN BCL-2 ACTIVITY WERE CALCULATED BY COMPARING DU WITH NU AND DVT AND DNT WITH DU MICE. [a] Statistically significant difference (*P*< 0.001) when compared with NU values. [b] Statistically significant difference (*P*< 0.001) when compared with DU values.

TUNEL Sssay

TUNEL Sssay in Heart Tissue: As compared to the number of TUNEL-positive cells in the heart tissue of normal mice (NU), which is 25%, there is a dramatic increase in the number of TUNEL-positive cells in the brain tissue of streptozotocin-treated mice (DU) to 87%. In the heart tissue of diabetic mice, however, vitamin C and naringenin administration for 28 days reduced apoptosis by 34% and 37%, respectively **Fig. 8A and 8B.**

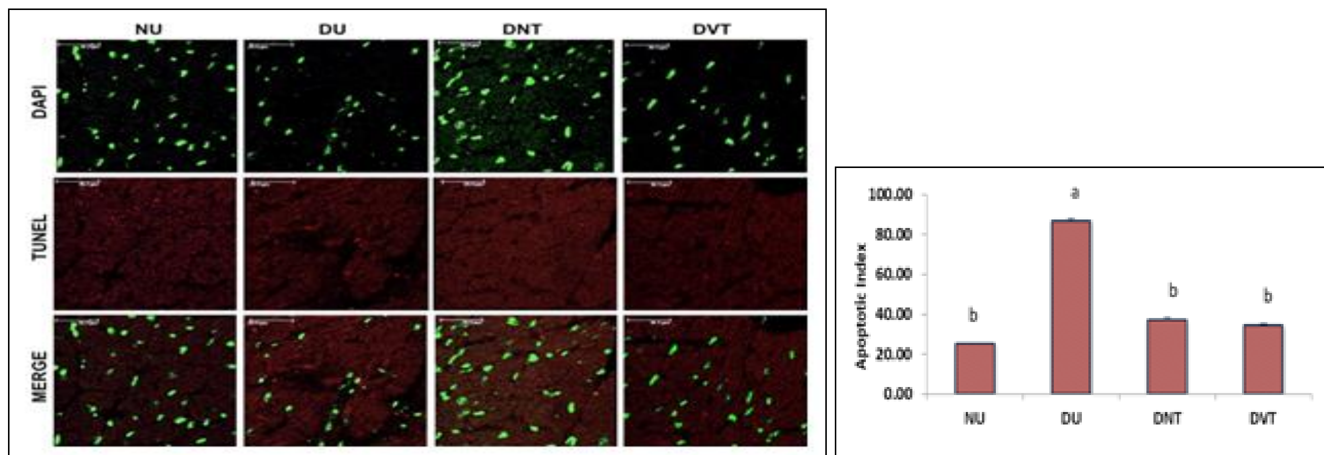

FIG. 8A: THE RESULT SHOWS DAPI, TUNEL, AND MERGE (DAPI+TUNEL) CONFOCAL MICROSCOPIC IMAGES OF HEART TISSUE OF EXPERIMENTAL GROUPS. DAPI-STAINED NUCLEI ARE SHOWN IN FLUORESCENT GREEN, WHEREAS TUNEL-POSITIVE APOPTOTIC CELLS ARE IN FLUORESCENT RED. SCALE BAR: 30MM. VALUES ARE EXPRESSED AS THE MEAN ± SEM. (B): THE RESULT SHOWS APOPTOTIC INDEX OF EXPERIMENTAL GROUPS. VALUES ARE EXPRESSED AS THE MEAN ± SEM. [a]Statistically significant difference ($P < 0.001$) when compared with NU values. [b]Statistically significant difference ($P < 0.001$) when compared with DU values.

TUNEL assay in Brain Tissue: As compared to the number of TUNEL-positive cells in the brain tissue of Normal mice (NU) which is 25%, there is a dramatic increase in the number of TUNEL-positive cells in the brain tissue of streptozotocin-treated mice (DU) to 83%. In diabetic mice, however, vitamin C and naringenin administration for 28 days reduced apoptosis by 32% and 33%, respectively **Fig. 9A and 9B.**

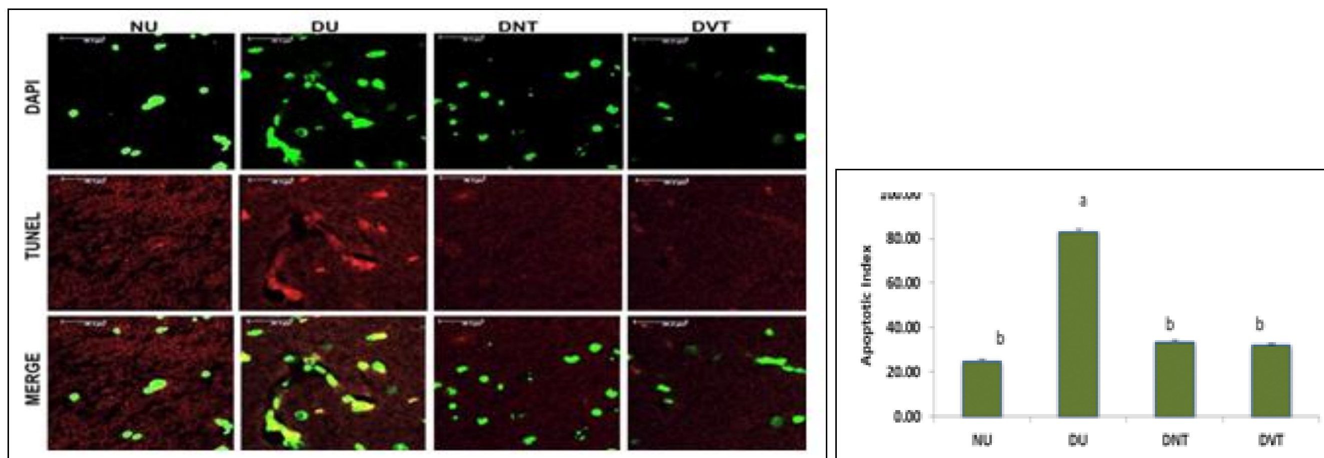

FIG. 9A: THE RESULT SHOWS DAPI, TUNEL, AND MERGE (DAPI+TUNEL) CONFOCAL MICROSCOPIC IMAGESOF BRAIN TISSUE OF EXPERIMENTAL GROUPS. DAPI-STAINED NUCLEI ARE SHOWN IN FLUORESCENT GREEN, WHEREAS TUNEL-POSITIVE APOPTOTIC CELLS ARE IN FLUORESCENT RED. SCALE BAR: 30MM. (B)THE RESULT SHOWS THE APOPTOTIC INDEX OF EXPERIMENTAL GROUPS. VALUES ARE EXPRESSED AS THE MEAN ± SEM. [a]Statistically significant difference ($P < 0.001$) when compared with NU values. [b]Statistically significant difference ($P < 0.001$) when compared with DU values.

DISCUSSION: In mouse models, experimental hyperglycemia is produced using the selective pancreatic cell toxin streptozotocin (STZ). Whereby it has a very high affinity for GLUT2

transporters on cells when administered in fasting condition [31]. Also, other cells that express this GLUT 2 transporter, such as the hepatocytes and the renal tubular cells, are also susceptible to STZ [32]. One of the mechanisms of STZ in β-cells damage is the generation of nitric oxide, which further reacts with superoxide ($O_2^{\cdot-}$) to generate other free radicals. This ROS elevation causes permanent oxidative damage to cells resulting in insulin deficiency and hyperglycemia. Metabolic pathways such as the polyol pathway, protein kinase C, and advanced glycation end-products (AGEs) formation are known to be activated under hyperglycemia which eventually exacerbates oxidative stress by elevating ROS levels [33].

One of the consequences of oxidative damage is lipid peroxidation, which occurs when polyunsaturated fatty acids (PUFA) are harmed by ROS-producing aldehyde molecules like MDA, which is one of the most well-known and reliable markers to measure oxidative stress in clinical settings due to its high reactivity and toxicity [34]. It is evident in several studies which indicated lipid peroxidation exhibiting excess MDA levels in diabetic experimental models [22, 35].

Similarly, our study is in line with these studies exhibiting elevated MDA in diabetic untreated mice as compared to the normal mice. However, our study shows that by reducing the level of MDA in diabetic mice treated with naringenin, it support sits protective role, particularly, against oxidative stress. SOD and CAT play a key role in quenching ROS. In dismutation reactions catalyzed by SOD, $O_2^{\cdot-}$ is reduced to hydrogen peroxide (H_2O_2), which further breaks into H_2O and O_2 in CAT-mediated reactions. In diabetes, several studies showed contradictory results relating to the elevation or diminution in the activity of these enzymes, which could be due to the variability in used diabetic models, the magnitude of insulin deficiency, and the duration of diabetes [36]. In our study, diabetic untreated mice showed a downgrading trend in the activity of such enzymes. This can be attributable to these anti-oxidant enzymes' free radicals- and/or persistent hyperglycemia-mediated glycation. In conjunction with activity, the present study also exhibited a significant decrease in SOD and CAT expression in the diabetic untreated group. As observed in our studies, treatment of naringenin in diabetic mice shows very promising outcomes with increased activity of the anti-oxidative enzyme such as SOD, CAT, GPX, and GST. The DNA is altered by the prolonged oxidative stress in the hyperglycemic state, which can cause harm to the nuclear DNA (nDNA) and mitochondrial genetic material (mtDNA) [37]. Further activation of the oxidative stress-diabetes interaction results in increased mitochondrial membrane permeability and apoptosis, which lowers immunity in diabetic patients and increases mortality and morbidity [38].

By changing the delicate balance between the expression of pro-apoptotic and anti-apoptotic proteins, high levels of this reactive species ultimately cause an increase in pro-apoptotic events. High levels of this reactive species play an important role in increased stimulation of the pathway that promotes apoptosis. The relative expression of these proteins is a crucial factor in determining how they ultimately affect cell fate. The stimulation of pro-apoptotic processes is crucial for the emergence of diabetic complications.

The improved activity of these anti-oxidative enzymes can be correlated with the result obtained from the apoptotic studies in which diabetic mice treated with naringenin show decreased over-expression of apoptotic executioner proteins such as caspase 3 and caspase 9, while increasing the expression of anti-apoptotic protein Bcl-2 in both the heart and the brain tissues, which is in complete contrast to that in the diabetic control mice. TUNEL assay confirms the above studies in which a lot of fragmented DNA can be observed in diabetic mice; however, naringenin herbal treatment of the diabetic shows decreased the number of fragmented DNA. Under continuous exposure to hyperglycemia-induced oxidative stress, the tissues undergo many changes and alterations in their structure in the process, losing their integrity, and this ultimately causes their dysfunction. Ultrastructural studies using TEM on all groups of mice normal (NU), diabetic untreated (DU), ascorbic treated (DVT), and naringenin treated (DNT) in the heart and brain tissues were analyzed. As observed from the above figure **Fig. 4A** and **4B** the heart and brain tissue in diabetic mice untreated undergoes a lot of ultrastructural alterations and damages; however, naringenin

treatment shows improved tissue architecture in both the heart and the brain. Naringenin supplementation, therefore, has a protective impact on the tissues, in addition to its anti-oxidant and anti-apoptotic effects. Due to the interdependence of the metabolism of carbohydrates and lipids, blood lipid levels can be influenced by various variables in people with diabetes. As a result, every issue in lipid metabolism causes a disorder in glucose metabolism, and vice versa [39]. High total cholesterol (T-Chol), high triglycerides (Tg), low levels of high-density lipoprotein cholesterol (HDL-C), and an increase in microscopic dense LDL particles are all symptoms of lipid abnormalities, often known as "diabetic dyslipidemia." [40]. Better management and correction of abnormal lipid profiles positively impact patients with diabetes. As observed in this study, naringenin treatment shows an overall improvement in the lipid profile of diabetic mice. Thus, with all these positive and promising effects, naringenin can definitely be a very potent and effective therapeutic adjuvant to attenuate hyperglycemia-induced oxidative stress and apoptosis.

CONCLUSION: Overall, our results show that STZ induction encourages the decline of antioxidant enzyme levels produced in the diabetes condition in the mice's heart, and brain tissue. However, naringenin treatment reduced the levels of oxidative stress brought on by hyperglycemia by activating and stimulating the various anti-oxidant enzymes. Also, it escalated the translocation of Bcl-2 to the mitochondria, partially preventing the release of mitochondrial cytochrome-C to the cytoplasm and ultimately preventing the activation of caspase-3 and caspase-9, producing a slight anti-apoptotic effect. Naringenin may thus help lower different aspects of diabetes-related issues in the heart, and brain of STZ-induced diabetic mice through a range of possible mechanisms through its anti-hyperlipidemia, antioxidant, and anti-apoptotic activities. Further investigation is required to identify the molecular mechanisms and demonstrate the therapeutic efficacy in humans.

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Consent for Publication: Not applicable

Availability of Data and Material: All data generated or analyzed during this study are included in this published article."

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