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Apigenin ameliorates diabetic neuropathy in rats by modulating the TLR4/MyD88 signaling pathway

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ABSTRACT

Objective: To determine the neuroprotective effects of apigenin against streptozotocin (STZ)-induced diabetic neuropathy (DN).

Methods: To induce DN, Wistar rats (150-200 g) were administered with STZ (55 mg/kg, *i.p.*). Then they were randomly assigned to various groups, *viz.*, normal, diabetic control, insulin (10 IU/kg, *s.c.*), apigenin (5, 10, and 20 mg/kg, *p.o.*), and insulin (10 IU/kg) plus apigenin (20 mg/kg, *p.o.*). Various behavioral, biochemical, and molecular markers [tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, Toll-like receptor 4 (TLR4), myeloid differentiation primary response 88 (MyD88), and nuclear factor erythroid 2-related factor 2 (Nrf2)] were assessed.

Results: Apigenin (10 and 20 mg/kg, *p.o.*) substantially reduced plasma glucose levels, lipid profile, aspartate transaminase, alanine transaminase, glycated hemoglobin, and neural advanced glycation end products in STZ-induced DN rats ($P<0.05$). After apigenin intervention, STZ-induced changes in food and water intake, body weight, urine output, allodynia, hyperalgesia, and insulin levels were markedly improved ($P<0.05$). Neural antioxidant enzymes (superoxide dismutase and glutathione) and Na$^+$K$^+$ATPase activity were also considerably elevated ($P<0.05$) while the level of lipid peroxidation was diminished following apigenin therapy ($P<0.05$). Furthermore, apigenin markedly upregulated the *Nrf2* mRNA level while downregulating the mRNA expressions of *TNF−α* and *ILs* and the protein expressions of TLR4 and MyD88 ($P<0.05$). STZ-induced histological abnormalities in the sciatic nerve were also improved by apigenin treatment.

Conclusions: Apigenin exerts its neuroprotective effect by modulating the inflammatory and oxidative stress pathways *via* regulating the TLR4-MyD88 signaling pathway.

KEYWORDS: Apigenin; Diabetic neuropathy; MyD88; Nrf2; Proinflammatory cytokines; TLR4

1. Introduction

Diabetes affects over 500 million individuals worldwide, and its prevalence is rising rapidly, with 700 million expected by 2045[1]. Diabetic late sequelae are classified as macrovascular complications, which include cardiovascular illnesses and microvascular problems comprising retinopathy, nephropathy, and neuropathy[2]. The most prevalent secondary consequence is diabetic neuropathy (DN), caused by impaired lower limb sensory function followed by pain and significant morbidity. Without proper treatment, one-third of the 9.7 billion people predicted to live in 2050 are expected to have diabetes, with half developing neuropathy[3]. In China, the prevalence of type 2 diabetes mellitus (T2DM) with DN was reported to be 57.2%, and the annual direct medical cost was reported to be ¥ 28 822.7 ($ 4 612) per person[4].

Significance

Apigenin, a flavonoid compound, shows antioxidant, anti-inflammatory, and antidiabetic properties. In the present study, apigenin alleviates streptozotocin-induced diabetic neuropathy by inhibiting elevated inflammatory release (TNF-α, IL-1β, and IL-6) and oxidative stress *via* regulating the TLR4-MyD88 signaling pathway. These findings suggest that apigenin may be a promising neuroprotective agent in diabetic neuropathy.

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The etiology of DN is characterized by poor peripheral nerve fiber and microvessel dysfunction. This is usually caused by hyperglycemia and other metabolic factors, such as hyperlipidemia and inadequate insulin signaling mechanisms, resulting in various subsequent pathogenic pathways[5]. Hyperglycemia promotes an increase in the Toll-like receptor 4/myeloid differentiation primary response protein 88 (TLR4/MyD88) signaling pathway, triggers inflammation, and accelerates the onset of nephropathy and peripheral neuropathy in diabetic rats[6].

The clinical challenge of controlling DN continues to persist despite therapeutic advancements. Research on behavioral and biomarker alterations offers early detection of these consequences for particular therapies that are helpful to avoid amputation which in turn decreases physical incapacity and mental disturbance[7]. Benfotiamine, acetyl-*L*-carnitine, α-lipoic acid, and methylcobalamin are among the main therapies for managing DN. However, only a fraction of patients benefit from these medicines. Natural products are considered a superior treatment alternative to cure DN since they are safer than current medications with minimum side effects.

Numerous studies on diabetic neuropathic pain have examined polyphenols and flavonoids in light of their potentially beneficial antioxidant and anti-inflammatory activities[8]. Apigenin (4', 5,7-trihydroxy flavone; Supplementary Figure 1) is one of the most widely distributed flavonoids and exhibits several pharmacological and nutraceutical uses. Apigenin shows a broad spectrum of biological properties, including antioxidant, antidiabetic, cardioprotective, anti-inflammatory, antitumor, antihypertension, antibacterial, sedative, and anxiolytic effects. It also prevents neurodegenerative disorders, including amnesia and Alzheimer's disease[9,10]. The antidiabetic effect of apigenin is attributed to its diverse mechanism of action, including stimulating insulin secretion, suppressing the α-glucosidase and aldose reductase enzyme, scavenging reactive oxygen species (ROS), controlling the adenosine monophosphate-activated protein kinase (AMPK) pathways, inhibiting the sodium-glucose co-transporter-2, upregulating glucose transporter-1 expression, and enhancing glucose uptake and glucose transporter-4 expression[9]. Furthermore, studies have shown that apigenin-loaded solid lipid nanoparticles alleviate streptozotocin (STZ)-nicotinamide administration-induced diabetes nephropathy by modulating the heme oxygenase-1/nuclear factor erythroid 2-related factor 2 (Nrf2)/nuclear factor kappa B (NF-κB) signaling pathway[11]. Wu *et al*. reported that apigenin has antihyperalgesic and antiallodynic effects with key involvement of the spinal 5-hydroxytryptamine receptor 1A as targets for the intervention of neuropathic pain[12]. Although apigenin has potential antidiabetic activities, no attempt has been made to examine its mechanism against DN. Thus, the current study aimed to explore the effect of apigenin on STZ-induced DN by performing behavioral, biochemical, electrophysiological, and histological analyses.

2. Materials and methods

2.1. Experimental animals and care

The animal house of the First Affiliated Hospital of Nanchang University supplied male Wistar rats (150-200 g), which were maintained at the following conditions: temperature: $(24 \pm 1)\,°C$, dark/light cycle: 12:12 h; relative humidity: 45%-55%, standard pellet chow, and *ad libitum* water.

2.2. Induction of diabetes and treatment

Diabetes was induced using STZ (Sigma Chemical Co., St Louis, MO, USA; 55 mg/kg, *i.p.*) according to previously reported methods elsewhere[6,11] and confirmed after 48 h of STZ injection by estimating plasma glucose levels using an enzymatic glucose oxidase peroxidase diagnostic kit (SD Fine Chemicals, Mumbai, India). The rats with plasma glucose levels >250 mg/dL were used for the present study. Then, after 4 weeks, rats were randomly divided into the following group (n = 15) *viz.*, diabetic (STZ) control (treated with double distilled water at a dose of 10 mg/kg), apigenin (purity: ≥95%; Sigma Chemical Co., St Louis, MO, USA; treated at the doses of 5, 10 and 20 mg/kg)[13], insulin (treated at a dose of 10 IU/kg, *s.c.*) and combination of apigenin (20 mg/kg) and insulin (10 IU/kg, *s.c.*). Another group of normal rats was maintained separately and treated with double distilled water. Rats were treated for the next 4 weeks, and various behavioral parameters were assessed.

2.3. Behavioral estimations

Various behavioral parameters were assessed including body weight (using an animal weighing balance), water intake, food intake, and urine output using a metabolic cage (Techniplast, Italy), mechanical hyperalgesia (Randall-Selitto paw pressure test; UGO Basile, SRL Biological Research Apparatus, Italy)[14], mechano-tactile allodynia (Von Frey hair test, IITC, Woodland Hills, USA)[15], thermal hyperalgesia (tail immersion test)[16], nerve conduction velocity [motor nerve conduction velocity (MNCV) and sensory nerve conduction velocity (SNCV); AD Instrument Pvt. Ltd., Lab Chart 7.3, Australia][17,18] were assessed at different timepoint.

2.4. Biochemical estimations

At the end of the study (8th week), a retro-orbital puncture technique was used to withdraw the blood, and the serum was separated to estimate the following biochemical parameters.

2.4.1. Serum biochemistry

The levels of serum triglyceride, total cholesterol, high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and very low-density lipoprotein-cholesterol (VLDL-C),

alanine transaminase (ALT), aspartate transaminase (AST), and glycated hemoglobin (glycated-Hb) were estimated using reagent kits (Accurex Biomedical Pvt. Ltd., India).

2.4.2. Serum insulin

Serum insulin was measured using a rat-specific insulin ELISA kit (Mercodia AB, Sweden).

2.4.3. Determination of oxidative stress, advanced glycation end products (AGEs), and inorganic phosphate in sciatic nerve

Rats were sacrificed under deep anesthesia using urethane (1000 mg/kg, *i.p.*), and sciatic nerves were immediately isolated. Tissue homogenate was prepared (0.1 M Tris-HCl buffer, pH 7.4), and the supernatant was used to determine levels of superoxide dismutase (SOD), reduced glutathione (GSH), lipid peroxidation [LPO; malondialdehyde (MDA) content], AGEs, and membrane-bound inorganic phosphate ($Na^+K^+ATPase$)[17].

2.4.4. Determination of inflammatory markers (TNF−α and ILs) and Nrf2 in sciatic nerve by reverse transcriptase polymerase chain reaction (RT−PCR), and TLR−4 and MyD88 by Western blot assay

The mRNA expressions of tumor necrosis factor-alpha (*TNF−α*), interleukins (*IL−1β* and *IL−6*), and *Nrf2* were estimated using RT-PCR (MP Biomedicals India Private Limited, India) according to the method described elsewhere[17]. The primer sequences for *TNF−α*, *IL−1β*, *IL−6*, *Nrf2*, and *β−actin* are provided in Supplementary Table 1. The protein expressions of TLR4 (40C1285, ab13915, 1:500, Abcam, Cambridge, MA, USA), MyD88 [EPR590(N), ab133739, 1:10000] and GAPDH (glyceraldehyde 3-phosphate dehydrogenase; EPR16891, ab181602, 1:1000) were estimated using Western blot assay according to previously reported methods[19].

2.4.5. Histological analysis of sciatic nerve

Another sample of the sciatic nerve (*n*=3) was subjected to histopathological analysis. Samples of sciatic nerve were placed in the fixative solution (10% formalin), cut into 5 μm thickness and stained with hematoxylin and eosin (H&E). The severity of sciatic nerve under a light microscope (Olympus DP71, DP-BSWVer.03.03, Olympus Medical Systems India Private Limited, India) (magnification: 100×) for neural damage, including neuronal degeneration, necrosis, and inflammatory infiltration, was scored as: 0 (none), 1 (minimal), 2 (mild), 3 (moderate) and 4 (severe)[20].

2.5. Statistical analysis

Data were expressed as mean with standard error of mean (SEM) or median with quartile range. Graph Pad Prism 5.0 software (Graph Pad, San Diego, USA) was used for analysis. Data from behavioral tests were analyzed by two-way analysis of variance (ANOVA), and

Bonferroni's multiple range test was applied for *post hoc* analysis. Whereas one-way ANOVA was used to analyze data on biochemical parameters, Tukey's multiple range test was applied for *post hoc* analysis. Data of histological score was examined by the Kruskal-Wallis test followed by Mann-Whitney's multiple comparison tests. A value of *P* <0.05 was considered to be statistically significant.

2.6. Ethical statement

The IAEC approved the experimental protocol (approval no. 20230307). The guidelines and regulations provided by the National Institute of Health Guide for Care and Use of Laboratory Animals were followed to perform all experiments.

3. Results

3.1. Effect of apigenin on body weight, plasma glucose level, food intake, water intake, and urine output

Three days before diabetes induction, both the normal (non-diabetic) and diabetic rats showed no significant (*P*>0.05) alterations in body weight or plasma glucose level. STZ administration resulted in a significant reduction (*P*<0.05) in the body weight of the rats and noticeable increases (*P*<0.05) in plasma glucose levels compared with the normal group. Apigenin (10 and 20 mg/kg) and insulin (10 IU/kg) treatment substantially (*P*<0.05) increased body weight and decreased plasma glucose levels in STZ rats. Furthermore, diabetic rats treated with both apigenin (20 mg/kg) and insulin (10 IU/kg) showed a prominent inhibitory effect (*P*<0.05) on decreased body weight and increased plasma glucose levels in comparison with apigenin alone treated rats (Figure 1A and B).

In addition, the diabetic control group exhibited a substantial (*P*<0.05) increase in food and water consumption (*P*<0.05) and urine output compared with the normal group. Apigenin (10 and 20 mg/kg) significantly (*P*<0.05) reduced urine output and food and water intake of diabetic rats. More importantly, a combination of apigenin (20 mg/kg) and insulin (10 IU/kg) showed more pronounced (*P*<0.05) improvement in food and water intake and urine output than apigenin alone treatment (Figure 1C-E).

3.2. Effect of apigenin on mechano−tactile allodynia and hyperalgesia

Paw withdrawal threshold (PWT) did not differ significantly (*P*>0.05) between diabetic control rats and normal rats one day before diabetes induction. However, PWT significantly declined (*P*<0.05) in diabetic rats from day 14 to 56. Treatment with apigenin (10 and 20 mg/kg) significantly (*P*<0.05) ameliorated the decrease in PWT. Furthermore, a combination of apigenin (20 mg/kg) and insulin (10 IU/kg) alleviated the decreased PWT more substantially

(*P*<0.05) than apigenin alone treatment (Figure 2A and B).

3.3. Effect of apigenin on thermal hyperalgesia

There was no significant (*P*>0.05) difference in tail withdrawal latency (TWL) between normal and diabetic control rats one day before diabetes induction. However, a noticeable decrease (*P*<0.05) in TWL was observed in diabetic control rats. Apigenin (10 and 20 mg/kg) and insulin (10 IU/kg) administered to rats substantially

(*P*<0.05) inhibited diabetes-induced decreased TWL compared to diabetic control rats. Furthermore, compared to apigenin alone treated rats, combination therapy with apigenin (20 mg/kg) and insulin (10 IU/kg) showed markedly improved (*P*<0.05) thermal hyperalgesia (Figure 2C).

3.4. Effect of apigenin on SNCV and MNCV

There was no significant (*P*>0.05) difference in SNCV and
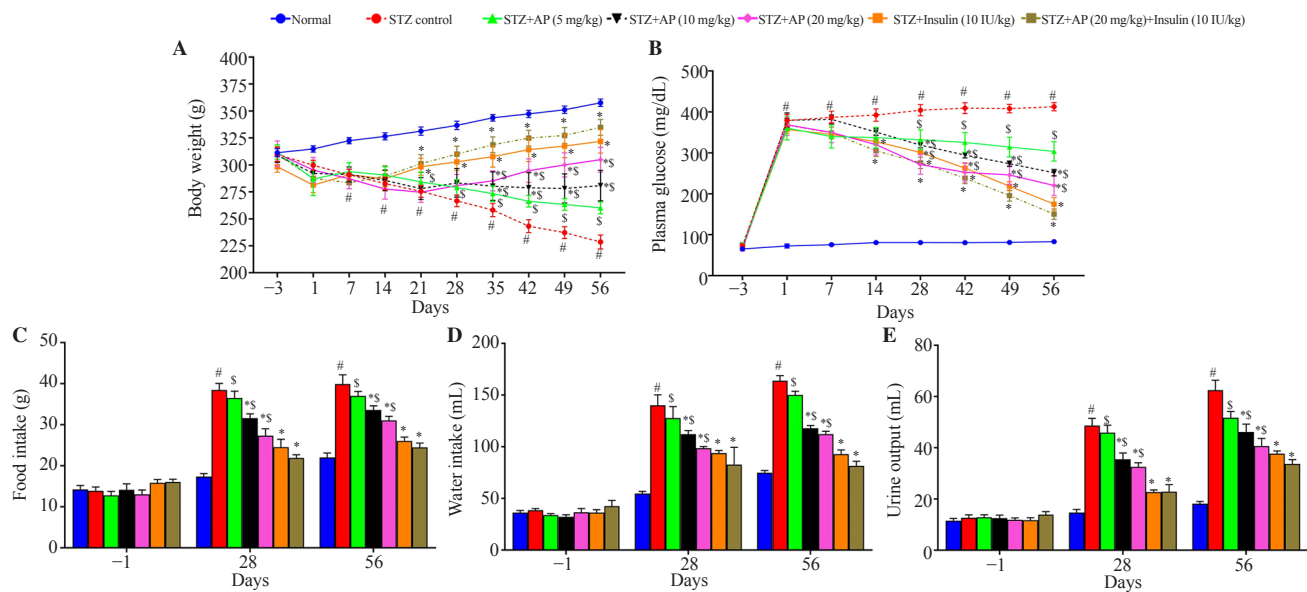

Figure 1. Effect of apigenin on diabetes-induced alterations in body weight (A), serum glucose (B), food intake (C), water intake (D), and urine output (E). Data are expressed as mean ± SEM (*n*=6) and analyzed by two-way ANOVA followed by Bonferroni's test. *P*<0.05 *vs.* STZ diabetic control, *P*<0.05 *vs.* normal control and *P*<0.05 *vs.* the AP+Insulin group. AP: apigenin; STZ: streptozotocin.
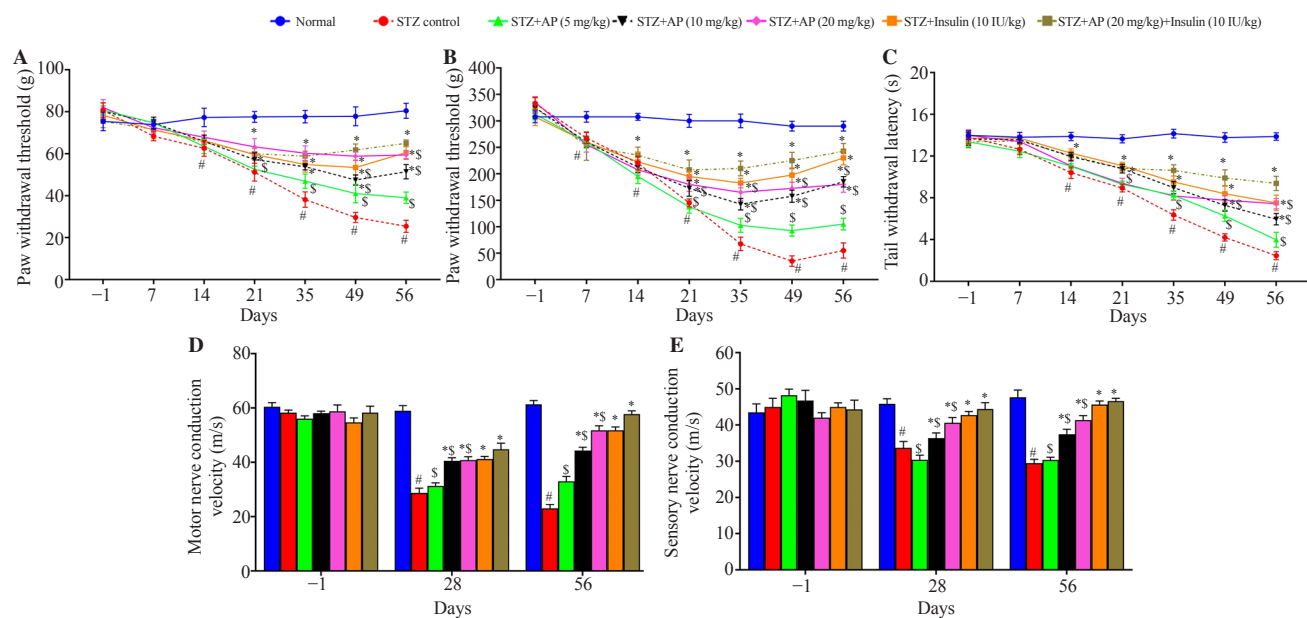

Figure 2. Effect of apigenin on diabetes-induced alterations in mechanical allodynia in von Frey hair test (A), mechanical hyperalgesia in paw pressure test (B), thermal hyperalgesia in plantar test (C), motor nerve conduction velocity (D), and sensory nerve conduction velocity (E). Data are expressed as mean ± SEM (*n*=6) and analyzed by two-way ANOVA followed by Bonferroni's test. *P*<0.05 *vs.* STZ diabetic control, *P*<0.05 *vs.* normal control and *P*<0.05 *vs.* the AP+Insulin group.

MNCV between the diabetic control and normal groups. However, intraperitoneal administration of STZ produced a marked ($P<0.05$) reduction in SNCV and MNCV in rats. The decrease in SNCV and MNCV was significantly ($P<0.05$) improved by apigenin (10 and 20 mg/kg) and insulin (10 IU/kg) intervention. Compared to apigenin alone treatment, treatment with both insulin and apigenin significantly ($P<0.05$) increased MNCV and SNCV (Figure 2D and E).

3.5. Effect of apigenin on serum lipid profile

Table 1 indicates that administration of STZ induced significant ($P<0.05$) alterations in the serum lipid profile of normal rats. Diabetic control rats had considerably ($P<0.05$) higher serum levels of cholesterol, triglycerides, LDL-C, and VLDL-C, while serum HDL-C levels were significantly reduced ($P<0.05$). Apigenin (10 and 20 mg/kg) treatment substantially ($P<0.05$) attenuated the STZ-induced changes in serum lipid profile. Additionally, combined treatment with insulin and apigenin significantly ($P<0.05$) mitigated the STZ-induced alterations in serum lipid levels compared with

apigenin alone treatment.

3.6. Effect of apigenin on serum AST, ALT, glycated Hb, insulin, and neural AGEs

In contrast to the normal control, diabetic control rats showed significantly increased AST, ALT, glycated Hb, and neural AGE, and decreased insulin level ($P<0.05$). Treatment with apigenin (10 and 20 mg/kg) resulted in a substantial ($P<0.05$) decrease in serum AST, ALT, glycated Hb, and neural AGE, and increased serum insulin in diabetic rats. Furthermore, combined treatment with apigenin (20 mg/kg) and insulin (10 IU/kg) markedly ($P<0.05$) decreased serum AST, ALT, glycated Hb, and neural AGE, and increased serum insulin compared with apigenin alone treatment (Table 2).

3.7. Effect of apigenin on neural oxidative stress

Administration of STZ resulted in an imbalance in the oxidative marker (LPO) and antioxidant markers (GSH and SOD). Diabetic control rats showed considerably higher ($P<0.05$) LPO

Table 1. Effect of apigenin on serum lipid profile in diabetes-induced neuropathic rats.

Parameter	Normal	STZ	STZ + AP (5 mg/kg)	STZ + AP (10 mg/kg)	STZ + AP (20 mg/kg)	STZ + Insulin (10 IU/kg)	STZ + AP (20 mg/kg) + Insulin (10 IU/kg)
LDL-C (mg %)	7.15 ± 1.00	18.87 ± 1.79#	19.47 ± 1.61$	15.03 ± 1.04*$	9.62 ± 0.95*$	7.92 ± 1.13*	7.94 ± 0.87*
HDL-C (mg %)	29.90 ± 2.15	8.07 ± 0.68#	9.85 ± 0.69$	14.06 ± 1.29*$	19.06 ± 1.29*$	24.84 ± 1.13*	27.85 ± 1.37*
Total cholesterol (mg/dL)	18.09 ± 1.07	43.30 ± 1.46#	39.25 ± 2.79$	37.53 ± 2.45*$	33.56 ± 3.84*$	29.40 ± 3.30*	21.25 ± 1.97*
Triglyceride (mg/dL)	77.82 ± 4.93	173.00 ± 15.12#	156.20 ± 6.68$	140.40 ± 6.68*$	124.80 ± 5.23*$	94.91 ± 2.63*	81.37 ± 12.14*
VLDL-C (mg %)	15.56 ± 0.99	34.59 ± 3.02#	31.24 ± 1.34$	28.09 ± 1.34*$	24.96 ± 1.05*$	18.98 ± 0.53*	16.27 ± 2.43*

Data are expressed as mean ± SEM (n=6) and analyzed by one-way ANOVA followed by Tukey's test. *$P<0.05$ *vs.* STZ diabetic control, #$P<0.05$ *vs.* normal control and $$P<0.05$ *vs.* the AP+Insulin group. STZ: streptozotocin; AP: apigenin; LDL-C: low-density lipoprotein-cholesterol; HDL-C: high-density lipoprotein-cholesterol; VLDL-C: very low-density lipoprotein-cholesterol.

Table 2. Effect of apigenin on serum AST and ALT, glycated hemoglobin, and insulin, as well as neural advanced glycation end-products in diabetes-induced neuropathic rats.

Parameter	Normal	STZ control	STZ + AP (5 mg/kg)	STZ + AP (10 mg/kg)	STZ + AP (20 mg/kg)	STZ + Insulin (10 IU/kg)	STZ + AP (20 mg/kg) + Insulin (10 IU/kg)
AST (IU/L)	63.81 ± 8.05	271.50 ± 14.11#	254.40 ± 9.43$	232.40 ± 15.71*$	218.60 ± 11.25*$	176.60 ± 21.68*	119.60 ± 11.09*
ALT (IU/L)	115.00 ± 13.94	371.20 ± 44.13#	354.50 ± 37.17$	318.40 ± 28.21*$	248.40 ± 23.72*$	201.80 ± 31.21*	171.90 ± 55.36*
Glycated Hb (%)	3.04 ± 0.31	14.72 ± 0.76#	12.51 ± 1.46$	10.47 ± 1.03*$	9.76 ± 0.72*$	8.18 ± 0.66*	3.35 ± 0.40*
Insulin (μg/L)	1.20 ± 0.06	0.51 ± 0.03#	0.58 ± 0.02$	0.74 ± 0.01*$	0.72 ± 0.06*$	0.81 ± 0.05*	0.90 ± 0.05*
Neural AGEs (AUF/ mg protein)	14.45 ± 1.11	60.98 ± 3.39#	56.50 ± 2.51$	52.65 ± 3.94*$	39.73 ± 3.73*$	32.88 ± 2.57*	30.58 ± 1.54*

Data are expressed as mean ± SEM (n=6) and analyzed by one-way ANOVA followed by Tukey's test. *$P<0.05$ *vs.* STZ diabetic control, #$P<0.05$ *vs.* normal control and $$P<0.05$ *vs.* the AP+Insulin group. AST: aspartate transaminase; ALT: alanine transaminase; Hb: hemoglobin; AGE: advanced glycation end-products.

Table 3. Effect of apigenin on neural SOD, GSH, LPO, and Na+K+ATPase in diabetes-induced neuropathic rats.

Parameter	Normal	STZ control	STZ + AP (5 mg/kg)	STZ + AP (10 mg/kg)	STZ + AP (20 mg/kg)	STZ + Insulin (10 IU/kg)	STZ + AP (20 mg/kg) + Insulin (10 IU/kg)
SOD (U/mg of protein)	22.68 ± 1.78	5.60 ± 0.87$	6.23 ± 0.89$	12.31 ± 1.08*$	14.37 ± 1.09*$	17.43 ± 1.95$	19.63 ± 1.80*
GSH (μg/mg protein)	1.53 ± 0.12	0.56 ± 0.11#	0.70 ± 0.10$	0.79 ± 0.09*$	1.12 ± 0.09*$	1.39 ± 0.13*	1.32 ± 0.10*
LPO (nM/mg of protein)	3.13 ± 0.58	10.70 ± 0.71#	10.24 ± 0.78$	8.19 ± 0.39*$	4.76 ± 0.49*$	4.62 ± 0.60*	3.23 ± 0.60*
Na+K+ATPase (μmol/mg of protein)	9.95 ± 0.35	1.83 ± 0.23#	2.55 ± 0.36$	4.43 ± 0.30*$	6.05 ± 0.24*$	8.17 ± 0.20*	8.86 ± 0.29*

Data are expressed as mean ± SEM (n=6) and analyzed by one-way ANOVA followed by Tukey's test. *$P<0.05$ *vs.* STZ diabetic control, #$P<0.05$ *vs.* normal control and $$P<0.05$ *vs.* the AP+Insulin group. SOD: superoxide dismutase; GSH: glutathione; LPO: lipid peroxidation.

concentrations and effectively decreased (*P*<0.05) GSH and SOD concentrations compared with normal rats. Compared to the diabetic control group, apigenin (10 and 20 mg/kg) treatment considerably (*P*<0.05) reduced the LPO levels and markedly (*P*<0.05) increased the GSH and SOD concentrations in sciatic tissue. However, more significant results in alleviating (*P*<0.05) the aberrant variation in the oxidative stress were observed when both apigenin (20 mg/kg) and insulin (10 IU/kg) were administered together to rats (Table 3).

3.8. Effect of apigenin on membrane–bound inorganic phosphate

Neural Na⁺K⁺ATPase activity in diabetic control rats was markedly reduced (*P*<0.05) compared with normal rats. Rats treated with apigenin (10 and 20 mg/kg) had significantly (*P*<0.05) higher activity of Na⁺K⁺ATPase. Additionally, combined treatment with apigenin (20 mg/kg) and insulin (10 IU/kg) markedly elevated the Na⁺K⁺ATPase activity (*P*<0.05) compared with treatment with apigenin alone (Table 3).

3.9. Effect of apigenin on TNF–α, ILs, and Nrf2 mRNA expressions

Diabetic control rats had substantially upregulated (*P*<0.05) mRNA expressions of sciatic *TNF–α* and *ILs*, and downregulated *Nrf2* mRNA expression compared with normal rats (*P*<0.05). Intervention with apigenin (10 and 20 mg/kg) significantly (*P*<0.05) downregulated the sciatic *TNF–α* and *ILs*, and upregulated (*P*<0.05) *Nrf2* mRNA expressions. Furthermore, as compared to apigenin alone treatment, a combination of apigenin (20 mg/kg) and insulin (10 IU/kg) significantly (*P*<0.05) mitigated these upregulated sciatic *TNF–α* and *ILs* mRNA expressions and downregulated (*P*<0.05) *Nrf2* mRNA expression (Figure 3).
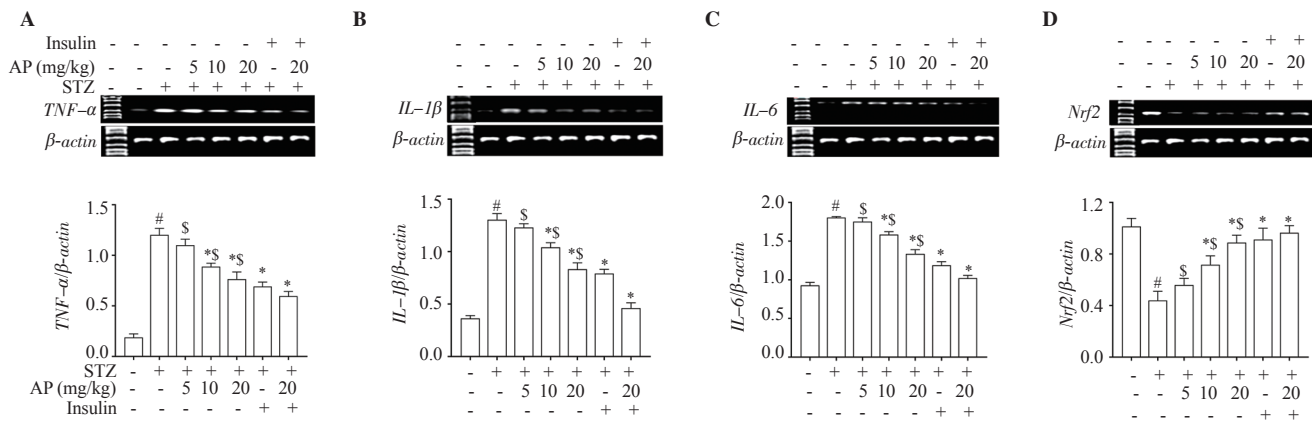

Figure 3. Effect of apigenin on diabetes-induced changes in mRNA expression of *TNF–α* (A), *IL–1β* (B), *IL–6* (C), and *Nrf2* (D). Data are expressed as mean ± SEM (*n*=6) and analyzed by one-way ANOVA followed by Tukey's multiple range test. *P*<0.05 *vs.* STZ diabetic control, #*P*<0.05 *vs.* normal control and $*P*<0.05 *vs.* the AP+Insulin group.
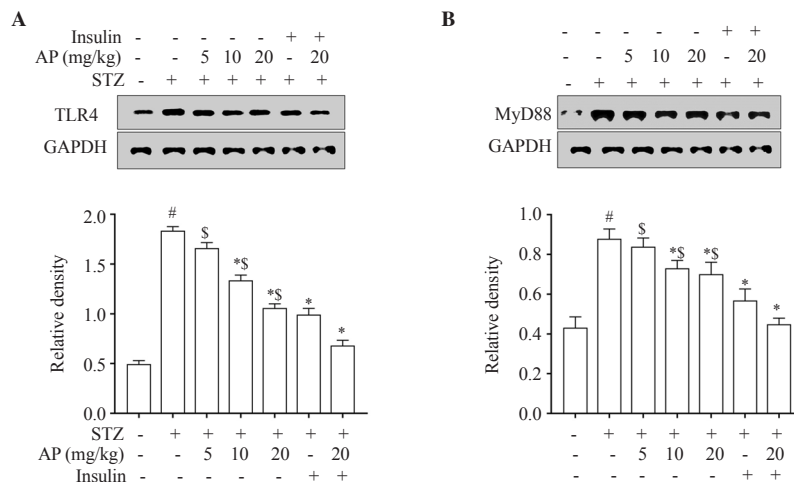

Figure 4. Effect of apigenin on diabetes-induced alterations in protein expression of TLR4 (A) and MyD88 (B). Data are expressed as mean ± SEM (*n*=6) and analyzed by one-way ANOVA followed by Tukey's multiple range test. *P*<0.05 *vs.* STZ diabetic control, #*P*<0.05 *vs.* normal control and $*P*<0.05 *vs.* the AP+Insulin group. TLR-4: Toll-like receptor 4; MyD88: Myeloid differentiation primary response 88.

3.10. Effect of apigenin on TLR4 and MyD88 protein levels

Diabetic control rats showed markedly ($P<0.05$) higher sciatic TRL4 and MyD88 protein levels in comparison with normal rats. However, apigenin (10 and 20 mg/kg) and insulin (10 IU/kg) treatment significantly ($P<0.05$) downregulated TLR4 and MyD88 protein levels in diabetic rats. Furthermore, these protein levels were markedly downregulated ($P<0.05$) in the group treated with both apigenin and insulin compared with the apigenin-treated group (Figure 4).

3.11. Effect of apigenin on sciatic nerve histology

Figure 5A displayed the normal architecture of the sciatic nerve tissue, where no evidence of neuronal degeneration, necrosis, edema, or inflammatory cell infiltration was observed. After 8 weeks of STZ administration, there was a substantial impairment ($P<0.05$) in the sciatic nerve tissue (Figure 5B), as evidenced by marked neuronal degeneration, infiltration of neutrophils and macrophage cells, congestion, necrosis, and edema of nerve cells. Furthermore, necrosis and vacuolization in the nerve cell caused inflammation of nonmyelinated and myelinated nerve fibers. All these abnormal histological changes were massively inhibited ($P<0.05$) by apigenin (10 and 20 mg/kg) (Figure 5C and Figure 5D) and insulin (10 IU/kg) (Figure 5E) compared with diabetic control rats. Furthermore, compared to apigenin alone treated groups, rats treated with insulin (10 IU/kg) and apigenin (20 mg/kg) demonstrated greater improvement ($P<0.05$) in the STZ-induced histological abnormalities in sciatic nerve (Figure 5F and Supplementary Table 2).

4. Discussion

DN is a diverse and complicated group of disorders causing abnormalities in electrophysiological function, represented by decreased nerve fiber conduction and loss of sensory and autonomic neural sensations. STZ-triggered DN in rodents has also been studied using behavioral indicators such as hyperalgesia caused by thermal, mechanical, and tactile allodynia in large sensory fibers and motor nerve fibers, which leads to activation of various biochemical and molecular pathways[21]. In the current study, apigenin inhibited elevated plasma glucose levels, TNF-α, ILs, TLR4, and MyD88 expressions, thus ameliorating STZ-induced allodynia and hyperalgesia.

In DN, behavioral responses to external stimuli are a promising predictor of altered sensation and pain[22]. Hyperalgesia and allodynia are neurological disorders caused by pathological changes in nerves due to an increased sensitivity to mechanical and thermal stimuli[23]. The aberrant clinical features of thermal pain perception such as hyperalgesia and allodynia were observed in STZ-induced painful DN[24]. Randall Selitto, Von Frey hair, and tail immersion tests have been reported as validated methods in rodents to assess the behavioral reactions of hyperalgesia and allodynia[25]. Research has shown that animals injected with STZ had allodynia and hyperalgesia[22]. Similar results were obtained in the current findings, where diabetic control rats receiving STZ displayed a lower paw and tail withdrawal threshold, which was ameliorated by apigenin.

Recent findings suggest that ROS develops DN and weakens the antioxidant defense system in T2DM patients[26]. When ROS (such as superoxide anion, hydrogen peroxide, and hydroxyl radicals) and reactive nitrogen species are persistently elevated and antioxidant activity declines, oxidative and nitrosative stress occurs, which is associated with endothelial dysfunction, insulin resistance, and
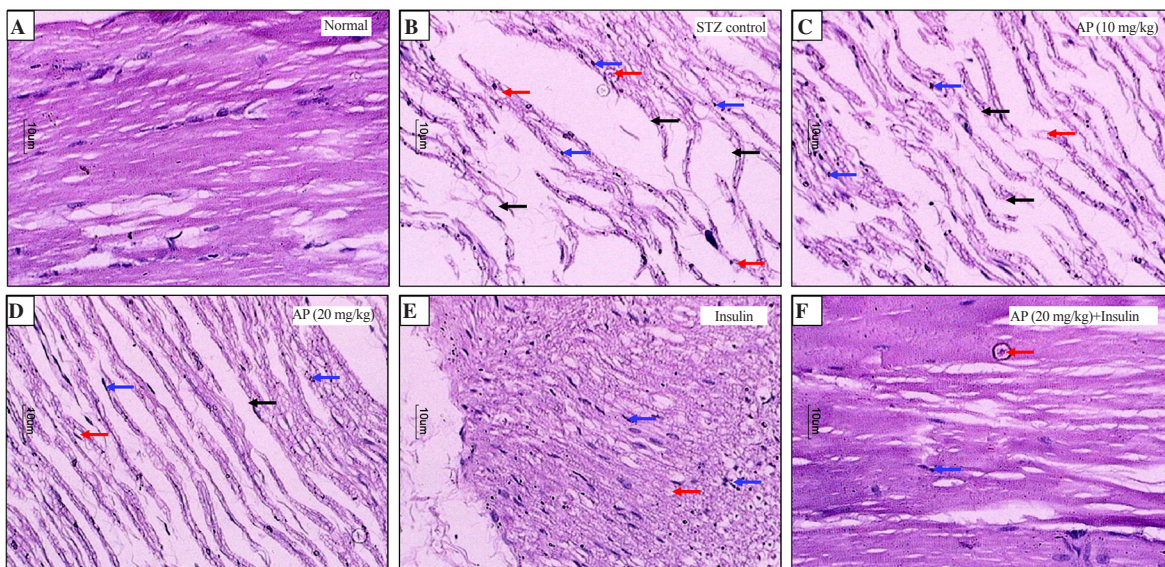

Figure 5. Effect of apigenin on diabetes-induced histopathological changes of sciatic nerve in rats. Photomicrographs of sciatic nerve sections from rats stained with H&E (100×). Necrosis (black arrow), inflammatory infiltration (blue arrow), and edema (red arrow).

changes in pancreatic function, ultimately leading to diabetic complications on the microvascular and macrovascular scales[27]. Neurons and vascular endothelium depend on SOD to maintain redox equilibrium, while GSH suppresses LPO within cells. On the other hand, MDA has been regarded as a key biomarker for oxidative stress and lipid damage caused by free radicals. Diabetes patients have been found to have higher levels of MDA in their serum and other tissues, which affect their peripheral nerves[21]. According to the research, flavonoids are natural antioxidants that significantly ameliorate DN-induced neuropathic pain by regulating the pathways of oxidative stress and inflammation[28]. Flavonoids, including catechin, genistein, luteolin, rutin, and pelargonidin, are potent antioxidants and have been shown to lower MDA levels in diabetic rats. Similar results were found in the current investigation, where diabetic control rats showed high MDA activity and reduced GSH and SOD activity in peripheral nerve tissue, and apigenin treatment effectively improved these oxidant-antioxidant imbalances. The findings of this investigation are consistent with the literature, where apigenin, apart from treating oxidative stress associated with DN, has also been reported as a potential antioxidant agent in various disorders such as diabetic nephropathy, cancer, Alzheimer's disease, Parkinson's disease, and spinal cord injury[29].

Nrf2 is a critical transcription factor with diverse biological roles in the cellular defense system by affecting the oxidative stress pathway. Nrf2 expression regulates a vital antioxidative defense mechanism and is involved in skin homeostasis[30]. The results of the present investigation revealed that apigenin upregulated Nrf2 expression in sciatic nerve tissue. Our findings align with earlier studies, suggesting that apigenin modulates adaptive responses to oxidants and electrophilic agents by upregulating Nrf2 signaling and enhancing cell antioxidant capacity[31].

Inflammation is a key pathogenic factor in DN, including changes in complex cellular pathways and processes[32]. In DN, peripheral nerve injury is accompanied by an inflammatory response at the site of damage, as well as upregulated proinflammatory mediators (TNF-α and ILs) in the serum and sciatic nerve of diabetic animals[33]. TNF-α is a proinflammatory mediator that has a significant role in the progression of DN, whereas IL-6 plays a dominant role in other microvascular complications, including nephropathy or retinopathy[34]. IL-1β, a proinflammatory cytokine, has been identified as a powerful cause of β-cell destruction, and investigations have confirmed that high levels of IL-1β are linked to DN[35]. According to the current findings, apigenin treatment mitigates the *TNF-α* and *ILs* mRNA overexpression caused by diabetes in the sciatic nerve. The literature revealed that apigenin also demonstrated significant anti-inflammatory activity in other experimental models, including lipopolysaccharide-induced inflammation[36], Alzheimer's disease, and anterior cruciate ligament transection-induced osteoarthritis[37]. These results support our findings, demonstrating that apigenin may function as a potential

anti-inflammatory agent by protecting the damaged sciatic nerve against edema, inflammatory cell infiltration, and necrosis.

Signaling pathways, including TLR4/MYD88, are crucial in controlling immunological and inflammatory responses[38]. Hyperglycemia promotes the activation of the TLR4/MyD88 signaling, triggers inflammation, and accelerates the onset of nephropathy and peripheral neuropathy in diabetic rats[6]. Thus, blocking the TLR4 signaling results in decreased TNF-α, IL-1β, and other inflammatory cytokines levels, leading to enhanced nerve function, such as mechanical hyperalgesia in rats with DN[39]. When ligands bind to TLR4, the key adaptor MyD88 is recruited as a dimmer to TLR4. Blocking the TLR4/MyD88 pathway decreases inflammatory markers and may play a role in developing novel medication to relieve DN in rats[39,40]. In the current study, apigenin prevents the upregulation of the TLR4/MyD88-dependent pathway in diabetic rats, which reduces inflammatory responses. Similar results were also reported in other studies, where apigenin attenuated the TLR4/MyD88 signaling to inhibit inflammatory response in animal models of allergic rhinitis, gouty arthritis, and oligosaccharide-induced inflammation in microglial cells[41,42].

Various clinical trials have revealed the potential of different therapeutic moieties of herbal origin in managing DN. In a randomized, double-blind study, oral curcumin supplementation attenuated inflammatory mediators like transforming growth factor-β and IL-8 in T2DM patients with neuropathy[43]. Although the pharmacological effects of apigenin have been examined extensively in pre-clinical research, relatively little information has been provided on its human clinical study. However, in a clinical assay involving a human cell line study conducted by Kang *et al.*, it was reported that apigenin showed neuroprotective effects by protecting and maintaining the viability of the SH-SY5Y human neuroblastoma cells against cell death induced by oxidative stress[44]. As a result, future clinical trials targeting DN should be promoted to leverage the medicinal uses of apigenin for human health.

The present study has some limitations. Firstly, during DN, a decrease in blood flow causes a reduction in oxygen levels and anaerobic metabolism. Thus, angiogenesis is another mechanism involved in DN that can be explored to evaluate the efficacy of apigenin. Secondly, DN is associated with structural modification in peripheral nerves, including fiber demyelination, axonal disjunction, and atrophy, which evolved slowly during the development of DN[5]. However, the potential of apigenin against DN-induced structural changes was not determined in the present investigation and can be further explored in future work.

In conclusion, the findings of the current study suggested that apigenin showed potential neuroprotective effects against diabetes-induced neuropathy by inhibiting elevated inflammatory release (TNF-α, IL-1β, and IL-6) and oxidative stress *via* modulating the TLR4/MyD88 signaling pathway.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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Data availability statement

The data supporting the findings of this study are available from the corresponding authors upon request.

Authors' contributions

YBY: Conception or design of the work, literature search, data analysis and interpretation, manuscript preparation, critical revision of the article, and final approval of the version to be published. MZQ: Conception of the work, data collection, manuscript editing and revision, critical revision of the article, and final approval of the version to be published. DYZ: Literature search, data analysis and interpretation, manuscript editing, and revision, critical revision of the article, and final approval of the version to be published.

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