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RESEARCH ARTICLE

Flavonoids aid in delaying the progression of diabetic neuropathy in type-2 diabetic rats

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Abstract

Diabetic neuropathy (DN), is a debilitating condition primarily synonymous with type-2 diabetes, which heightens the nociceptive response. This study examines the effects of hesperidin, a bioflavonoid, in combination with a flavonoid-rich methanolic extract of *Emblica officinalis* fruit (MEEF). A modified diet (high fat and fructose) was fed to male Wistar rats for a 14-day duration, along with a sub-diabetogenic dose of streptozotocin for DN induction. Diabetic rats were then dosed with MEEF, hesperidin (high 50% & low 25% dose), combination (low dose hesperidin + MEEF) and metformin (standard). Assessment of parameters such as serum glucose levels, antioxidant status, lipid profile, & TNF-α & IL-6 was conducted. Low-dose flavonoid treatment (hesperidin/ MEEF), known for its antioxidant effects, successfully reduced neuropathy-like symptoms (hyperalgesia) in diabetic subjects. This was shown by increased tail flick response times in hot plate and tail immersion tests compared to diabetic controls and metformin. Restoration of defensive antioxidative enzymes (superoxide dismutase and catalase) in treatment groups was observed, along with an improvement in lipid peroxidation. At the molecular level, the treatment also regulated IL-6 and TNF-α, which aided in recovery by neuroprotection. The findings indicated that combination therapy of hesperidin and MEEF evidently aided in alleviating neuropathic pain induced by hyperglycemia by delaying its progression.

Keywords: Hesperidin, *Emblica officinalis*, Diabetic neuropathy, Antioxidant, Neuroprotection.

Introduction

Diabetic neuropathy (DN), a common complication of type 2 diabetes, affects nearly half of diabetic patients globally (Smith *et al*., 2022; Galiero *et al*., 2023). The condition is characterized by nerve impairment, which leads to sensory, motor, and autonomic disturbances as a result of the degradation of sympathetic/parasympathetic conduction (Sacks *et al*., 2023). Elevated blood glucose levels activate the polyol pathway, increase oxidative stress due to the generation of reactive oxidative stress, cause nerve hypoxia, build up advanced glycation end products, enhance the engagement of the hexosamine pathway, and trigger protein kinase C (Akram *et al*., 2023; Pan *et al*., 2022; Pang *et al*., 2020).

Hesperidin, a bioflavonoid, has gained attention for its free radical scavenging, reduction of inflammatory mediators, and neuroprotection, particularly in a plethora of neurodegenerative conditions (Gandhi *et al*., 2020). Its antioxidant activity is especially relevant to DN, where oxidative stress plays a key role (Evans *et al*., 2022). Hesperidin scavenges free radicals, inhibits lipid peroxidation, and supports endogenous antioxidant systems, potentially reducing nerve damage, alleviating neuropathic symptoms, and lowering serum blood glucose levels (Syed *et al*., 2023; Ferraz *et al*., 2020). Additionally, hesperidin's anti-inflammatory effects may mitigate neuroinflammation, a contributor to the progression of DN. Hesperidin may help treat this condition by stopping the production of pro-inflammatory cytokines and reducing the activity of inflammatory pathways (Villarreal *et al*., 2020). Although preclinical studies are promising, clinical evidence remains limited, necessitating further investigation through larger randomized controlled trials to confirm its efficacy and safety. *Emblica officinalis* (amla) effectively countered arsenic-induced DN in mice by enhancing glucose regulation, lowering oxidative stress, and reducing inflammation (Singh *et al*., 2020).

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Exploring natural compounds like hesperidin and *E. officinalis* for their neuroprotective properties presents a promising approach to improving DN management.

The present study attempts to evaluate the effectiveness and safety of hesperidin and methanolic extract of *E. officinalis* fruit (MEEF) for managing DN, promoting their use as complementary therapies in clinical settings.

Methodology

Drugs and Chemicals

Streptozotocin (STZ) was purchased from Yucca Enterprises, Mumbai, Maharashtra, while hesperidin was sourced from Sigma-Aldrich, MO, USA.

Fructose Diet

In accordance with the approach described by Kumar *et al.* (2009), a diet consisting of fructose (600 g), protein (100 g), fat (80 g), vitamins (5 g), minerals (5 g), and cellulose (150 g) was prepared. All the nutrients were commercially procured from Bengaluru.

Extraction of Flavonoids

E. officinalis Gaertn fruit extract (flavonoid rich; methanolic fraction), was obtained from Green Chem Laboratories, Bengaluru, India. A methanolic extract was prepared by extracting 500 g of coarse dried fruit powder in a soxhlet apparatus with 2 L of methanol for 24 hours (three cycles). The mixture was then filtered, concentrated, and lyophilized (LyoQuest, Telstar, Spain) and dissolved in double-distilled water at desired concentrations for analysis (Middha *et al*., 2015).

Animals

The Institutional Animal Ethics Committee of Krupanidhi College of Pharmacy approved the study bearing Approval No. KCP/IAEC/PCOL/135/AUG-2023. Wistar rats that weighed between 200–250 g were selected & housed in favorable environmental conditions: temperature maintained at 22° ± 2°C, controlled humidity of 55 ± 5% & 12-hour light/dark cycle. A standard pellet diet with free access to water was provided before being switched to a high-fat diet.

Type 2 diabetes induction model: High Fat Diet and Low-Dose Administration of STZ

The rodents received a modified high-fat diet once daily for a two-week duration. After fasting overnight, they were treated with STZ (35 mg/kg/i.p) body weight solubilized in a 0.1 M citrate buffer with a pH of 4.4. Diabetes was diagnosed in rats with non-fasting plasma glucose levels exceeding 300 mg/dL. Vital fluid was drawn from the tail vein, and glucose levels were quantified with the AccuCheck diagnostic kit from India (Kumar *et al.,*2009; Srinivasan *et al.,* 2005).

Treatment Protocol

Following the recording of basal values in the diabetic rats, six animals were assigned to seven different groups. Normal and diabetic controls were dosed with the vehicle (0.5% Na-CMC) orally once daily. One group received MEEF (10 mg/kg, dissolved in 0.5% Na-CMC) orally once daily (Kumar *et al.,* 2009). Two supplementary groups received hesperidin (25 and 50 mg/kg, respectively, dissolved in 0.5% Na-CMC) (Mahmoud *et al*., 2012) orally once daily. The primary treatment group was given a combination of the low-dose hesperidin and the MEEF orally once daily. This combination was compared to the standard treatment group, which received 70 mg/kg metformin (MET) orally once daily (Zhang *et al.,* 2017). Drug solutions and extracts were prepared fresh and administered over a ten-week period, beginning one-week post-STZ administration.

Lipid Peroxidation (LPO) Measurement

The sciatic nerve was removed bilaterally and homogenized in 2.5% phosphate buffer saline at 7.0 pH in a polytron homogenizer post-incubation in Triton X-100 for 20 minutes. The amount of thiobarbituric acid reactive substances at a wavelength of 535 nm was used to measure lipid peroxidation. An extinction coefficient of 156 mmol/cm was used to find the malondialdehyde (MDA) concentrations in µM per mg of protein (Slater, 1984; Tang *et al*., 2019).

Superoxide Dismutase (SOD) and Catalase (CAT) Activity Measurement

Following centrifugation of the sciatic nerve homogenate at 17,500 g for 10 minutes at 4°C, the supernatant was utilized to measure SOD activity via the hematoxylin autooxidation technique, and the assessment of CAT was evaluated through a hydrogen peroxide (H_2O_2) degradation assay (Fridovich, 1975; Hadwan *et al.,* 2024).

Total Protein

Quantification of total protein followed Lowry's method (Lowry *et al.,* 1951).

Reduced Glutathione (GSH) Estimation

Nerve homogenate was mixed in equal parts with 10% trichloroacetic acid and centrifuged. The supernatant was incubated with phosphate buffer (pH 8.4), 5,5′-dithiobis (2-nitrobenzoic acid), and distilled water. After vortexing, absorbance at 412 nm was measured and reduced GSH was quantified in micrograms per milligram of protein (Tipple *et al*., 2012).

To assess Anti-nociception

Tail immersion test

The rats were plunged in water kept at 45° ± 1°C, followed by the recording of the withdrawal time with a cut-off of 15 seconds.

Eddy's hot-plate

Each rat was placed on the hot plate at 50° ± 1°C. The latency until paw grooming was noted as a tolerance level of pain,

with a maximum limit of 15 seconds imposed to prevent any injury to the paw (Cepeda-Benito *et al.*, 2000).

Histopathology

Sciatic nerve samples were preserved in 10% formalin. Samples were sliced into 4-micrometer sections. Sections stained with hematoxylin highlighted the cellular structures. Inspection of the stained sections with a light microscope at 100X magnification assessed the extent of axonal degeneration.

Statistical Analysis

Data is shown as mean ± standard error of mean (SEM), and an unpaired student's t-test was employed for comparisons between pairs of groups. For variations across groups, a one-way ANOVA was applied, and Tukey's post-hoc test was followed. The assessment of behavioral data employs a two-way repeated measures ANOVA, while biochemical data was analyzed using a one-way ANOVA with a subsequent Tukey's test. A *p-value* of ≤ 0.01 signifies statistical significance.

Results

Impact on Blood Glucose Levels

Figure 1 illustrates the impact of various treatments on blood glucose levels over a 10-week period. At week 3, all diabetic groups show elevated glucose levels. By week 6, MET and high-dose hesperidin (HDH) demonstrated significant reductions, with MET being the most effective. Low-dose hesperidin (LDH), *MEEF*, and their combination (LDH+MEEF) show moderate improvements. At week 10, MET, HDH, and LDH+MEEF led to the largest reductions.

Reduced GSH

Depleted GSH levels following diabetes induction were observed. However, post-ten weeks of treatment with the combination consequently elevated reduced GSH levels (p <0.01) as observed in Figure 2.

Lipid Peroxidation (LPO)

MDA activity exhibited a notable surge in the diabetic controls (p <0.05). However, the administration of HDH, LDH + MEEF, and MET caused a notable reduction in MDA levels (p <0.05). Combination treatment over ten weeks significantly lowered MDA levels in the serum (p <0.01) as shown in Figure 3.

SOD and CAT Activity Measurement

In diabetic rats at 10 weeks, SOD activity was significantly diminished relative to normal controls. Following combination therapy, a marked restoration of SOD activity to baseline levels was observed (p <0.001). Moreover, CAT activity was substantially elevated in untreated diabetic controls in comparison to their normal counterparts.

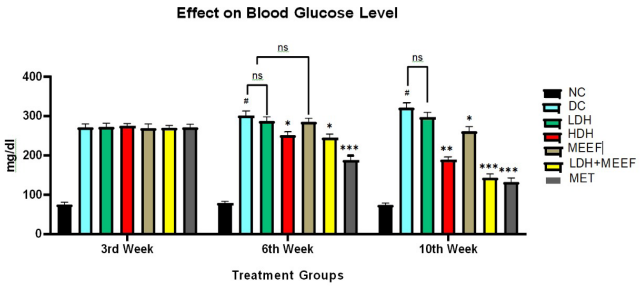


Figure 1: Impact of various treatments on blood glucose level. Mean ± SEM, n = 6, is used to represent data. ***p < 0.001, **p < 0.01, and *p < 0.5 are noted in relation to the diabetic control group, #p < 0.001 relative to normal control.

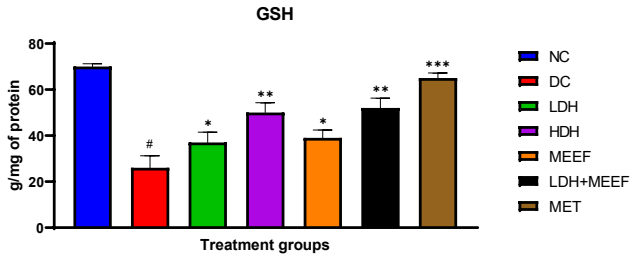


Figure 2: Impact of various treatments on GSH Levels. Mean ± SEM, n = 6, is used to represent data. ***p < 0.001, **p < 0.01, and *p < 0.5 are noted in relation to the diabetic control group, #p < 0.001 relative to normal control.

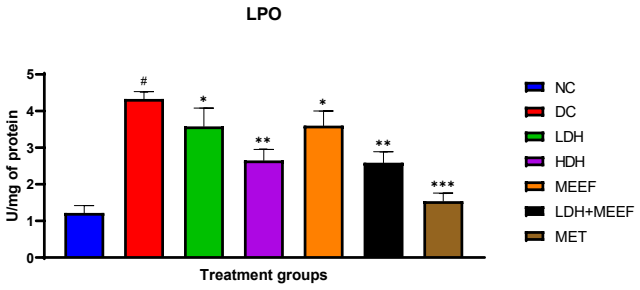


Figure 3: Impact of various treatments on LPO levels. Mean ± SEM, n = 6, is used to represent data. ***p < 0.001, **p < 0.01, and *p < 0.5 are noted in relation to the diabetic control group, #p < 0.001 relative to normal control.

The combination treatment over ten weeks effectively normalized CAT activity (p <0.01), and a notable intrinsic effect was evident in the combination group following the treatment period, as seen in Figure 4.

Total Protein

HDH and the combination therapy (LDH + MEEF) notably elevated protein levels (p <0.01). MET indicated the highest increase (p <0.001), emphasizing its effectiveness in improving glycemic control and management of DN as seen in Figure 5.

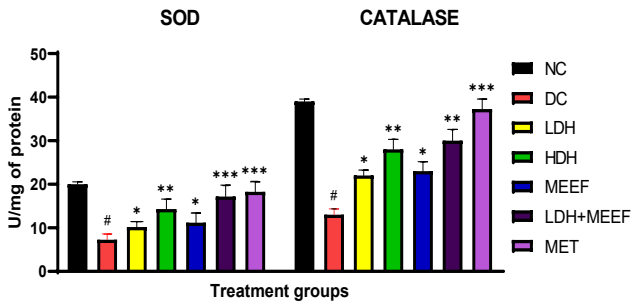


Figure 4: Impact of various treatments on SOD and CAT Activity. Mean ± SEM, n = 6, is used to represent data. ***p < 0.001, **p < 0.01, and *p < 0.5 are noted in relation to the diabetic control group, #p < 0.001 relative to normal control

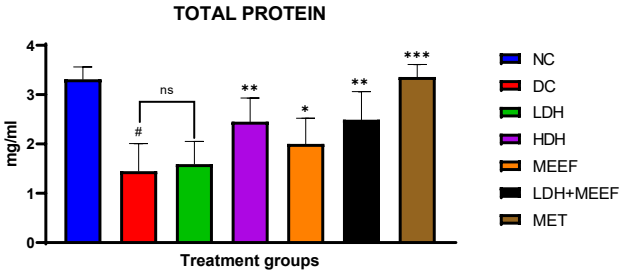


Figure 5: Impact of various treatments on Total Protein levels. Mean ± SEM, n = 6, is used to represent data. ***p < 0.001, **p < 0.01, and *p < 0.5 are noted in relation to the diabetic control group, #P< 0.001 relative to normal control

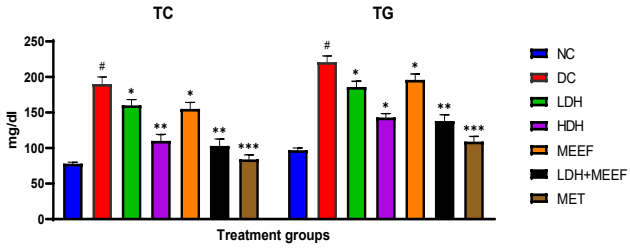


Figure 6: Impact of various treatments on TC and TG levels. Mean ± SEM, n = 6, is used to represent data. ***p < 0.001, **p < 0.01, and *p < 0.5 are noted in relation to the diabetic control group, #p < 0.001 relative to normal control

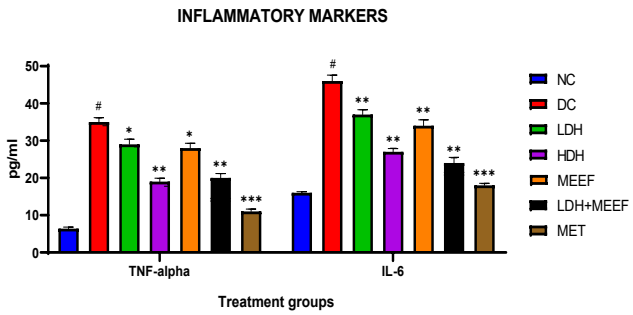


Figure 7: Impact of various treatments on Inflammatory markers. Mean ± SEM, n = 6, is used to represent data. ***p < 0.001, **p < 0.01, and *p < 0.5 are noted in relation to the diabetic control group, #p < 0.001 relative to normal control.

Impact on Total Cholesterol (TC) and Total Triglycerides (TG)

Following the *i.p.* administration of STZ, diabetic rats indicated a marked heightening of serum TC and TG levels relative to normal controls (p <0.05). When compared to diabetic controls, combination therapy (LDH and MEEF) and HDH showed significant drops in TC and TG (p <0.01). MET treatment alone showed the most notable decline (p <0.001) as depicted in Figure 6.

Impact on Inflammatory Markers

The levels of tumour necrosis factor-alpha (TNF-alpha) and Interleukin-6 (IL-6) were measured, showing that both cytokines were significantly elevated in diabetic rats compared to healthy rats (p < 0.001). The levels of the inflammatory markers went down after treatment with LDH and MEEF. However, the combination and HDH treatments led to a bigger drop in these cytokines compared to single treatments (p < 0.01), with MET treatment showing the biggest drop (p < 0.001) as shown in Figure 7.

Impact on Nociception

By the fourth week, the diabetic control group continues to deteriorate, while all treatments show positive effects. HDH and the combination of LDH and MEEF led to marked improvements continuing up to the 10th week, as shown in Tables 1 & 2.

Histopathology

Histopathology of sciatic nerve is shown in Figures 8 (a-d)

Discussion

Significant metabolic imbalances, such as elevated blood glucose levels (hyperglycemia), abnormal lipid profiles (dyslipidemia), and insulin resistance, exemplify DN (Baum *et al.*, 2021). Such conditions lead to an excess of reactive oxidative stress, thereby inducing oxidative stress and subsequently damaging the nerves. These pathophysiological alterations align with existing research, which considers oxidative stress to be a major contributor to the advancement of long-term complications linked to diabetes, particularly DN (Caturano *et al.*, 2023; Maldonado *et al.*, 2023).

In diabetic conditions, the increase in oxidative stress due to mitochondrial dysfunction leads to significant biochemical changes that disrupt nerve function (Zhang *et al.*, 2023). The present study affirms this by indicating higher levels of MDA, indicative of LPO, and reduced antioxidant enzyme activities, such as SOD and CAT. Previous research indicated that long-term hyperglycemia contributes to a

Table 1: Impact of various treatments on Eddy's hot plate (Reaction time in sec.)

Groups	*1st Week*	*4th Week*	*7th week*	*10th week*
Normal control	13.79 ± 0.41	13.87 ± 0.39	14.43 ± 0.23	14.75 ± 0.14
Diabetic control	10.31 ± 0.26#	7.78 ± 0.49#	6.98 ± 0.38#	6.12 ± 0.51#
LDH	9.43 ± 0.71	8.31 ± 0.34	8.39 ± 0.23*	8.87 ± 0.54**
HDH	10.18 ± 0.91	12.45 ± 0.29**	12.68 ± 0.40**	12.96 ± 0.26***
MEEF	9.39 ± 0.46	8.41 ± 0.74*	8.34 ± 0.36**	8.63 ± 0.28**
LDH+MEEF	9.26 ± 0.51	12.91 ± 0.23**	12.69 ± 0.61**	13.01 ± 0.63***
MET	10.12 ± 0.23	13.34 ± 0.93***	13.78 ± 0.86***	14.03 ± 0.28***

Mean ± SEM, n = 6, is used to represent data. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.5$ are noted in relation to the diabetic control group, #$p < 0.001$ relative to normal control.

Table 2: Impact of various treatments on tail immersion test (Reaction time in sec.)

Groups	*1st Week*	*4th Week*	*7th week*	*10th week*
Normal control	13.83 ± 0.38	13.77 ± 0.34	14.12 ± 0.76	14.47 ± 0.65
Diabetic control	8.98 ± 0.41#	7.96 ± 0.71#	6.67 ± 0.27#	5.07 ± 0.76#
LDH	8.67 ± 0.54	8.18 ± 0.41	7.87 ± 0.89*	8.53 ± 0.298**
HDH	9.23 ± 0.35	12.18 ± 0.17**	12.32 ± 0.30**	12.56 ± 0.21**
MEEF	9.41 ± 0.58	8.42 ± 0.38*	9.05 ± 0.58**	9.31 ± 0.718**
LDH+MEEF	9.47 ± 0.70	12.46 ± 0.19***	12.61 ± 0.65***	13.17 ± 0.76***
MET	10.44 ± 0.87	13.18 ± 0.64***	13.83 ± 0.67***	14.03 ± 0.08***

Mean ± SEM, n = 6, is used to represent data. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.5$ are noted in relation to the diabetic control group, #$p < 0.001$ relative to normal control.

weakened antioxidant defense system and subsequent nerve damage (Matough *et al*., 2012). The ability of hesperidin and MEEF to reverse these changes underscores their potential as therapeutic agents. The restoration of antioxidant activities and mitigation in MDA levels (Figure 3) after treatment with these flavonoids highlight their antioxidant properties (Figure 4), which are crucial in protecting the sciatic nerve from oxidative stress.

The neuroprotective effects of LDH and MEEF are strong, as shown by their ability to raise GSH levels (Figure 2) and restore antioxidant enzyme activity. GSH, a vital endogenous antioxidant, is particularly important in combating oxidative stress, and its depletion in diabetic animals correlates with nerve damage. The combination's ability to induce GSH regeneration suggests that it enhances endogenous antioxidant defense, thus mitigating the detrimental effects of hyperglycemia on nerve tissues. The flavonoid's capability of free radical scavenging provides a plausible mechanism for its observed neuroprotective effects (Bellavite, 2023).

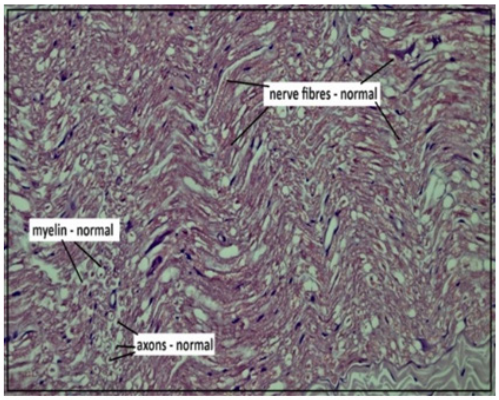
The study also highlights the role that lipid metabolism plays in the development of DN. It shows that dyslipidemia can make nerve damage worse on its own or in combination with hyperglycemia (Ozaki *et al*., 2018). The treatment reduced serum TG and TC (Figure 6), suggesting improved lipid utilization and metabolism. This is significant as it points to the dual role of the combination in not only reducing oxidative stress but also correcting lipid imbalances, which are critical contributors to DN. Previous research has also suggested that dyslipidemia may act synergistically with hyperglycemia to accelerate nerve degeneration (Rao *et al*., 2021).

Hesperidin and MEEF demonstrated significant antioxidant effects. However, the pronounced antinociceptive activity of LDH + MEEF, particularly from the fourth week onward (Tables 1 & 2), highlights its unique potential for mitigating DN. The elevation in pain thresholds eventually occurred as the duration of treatment continued. The results show that hesperidin and MEEF may have effects other than antioxidants. They may also change pain pathways and show better nociception over the course of ten weeks. This shows that the treatment might be able to stop the disease from getting worse altogether.

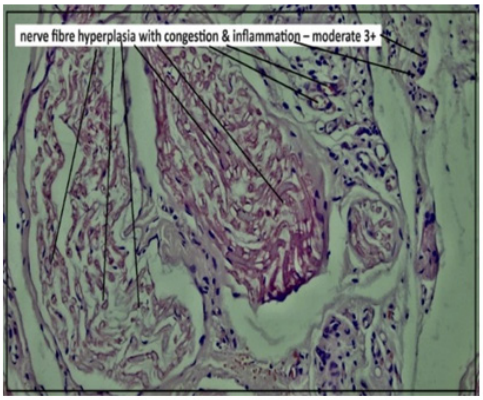
The neurotrophic role of nerve growth factor (NGF) in neuronal development and repair is well established. Diabetic conditions are known to reduce NGF levels, which contributes to DN progression (Shi *et al.,* 2018).

Also, there has been a noticeable change in TNF-alpha and IL-6 levels compared to diabetic controls (Figure 7). This supports the idea of neuroprotection by lowering inflammation in the sciatic nerve (Figure 8).

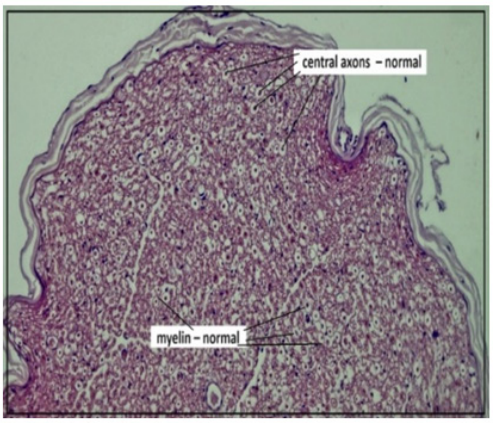
In conclusion, this research supports the idea that antioxidants such as MEEF and hesperidin can effectively



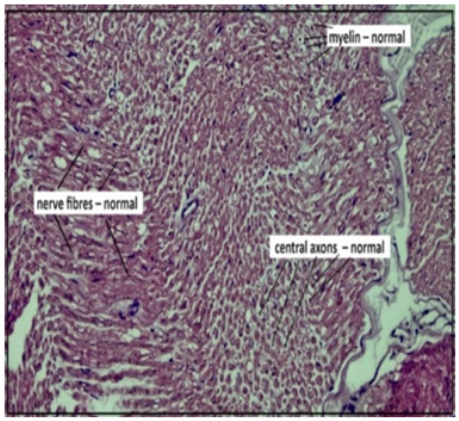
(a) Normal control- Sciatic nerve- showed normal neuronal architecture.



(b) Diabetic control- Sciatic nerve showed neuronal hyperplasia, inflammation with area of congestion.



(c) MET-Sciatic Nerve- Within normal limits.



(d) MEEF + LHD- sciatic nerve- showed normal architecture with low inflammation.

Figure 8: Histopathology of sciatic nerve

reduce oxidative stress, thereby halting the progression of DN. This is achieved through various mechanisms, such as inhibiting LPO, enhancing endogenous antioxidant defences, modulating NGF expression, and improving glucose and lipid metabolism, delaying the progression of DN. Supplementing these flavonoids through diet may aid in the hypoglycemic and neuroprotective effects, making them promising candidates for further exploration as preventive and therapeutic agents in DN and related complications.

Conclusion

This study offers compelling evidence that Hesperidin and MEEF possess significant neuroprotective potential against DN. By mitigating oxidative stress, enhancing antioxidant defenses, modulating NGF expression, and improving metabolic parameters, these compounds demonstrate their potential as promising therapeutic agents.

These findings emphasize the need to investigate natural substances in managing DN as they may aid in delaying disease progression. Clinical effectiveness in human patients needs to be examined. By deepening our knowledge of these natural antioxidants, dietary supplementation of these bioflavonoids, along with lifestyle modification, can prove to be an alternative to conventional therapy.

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