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ORIGINAL CONTRIBUTION

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Prevention of Chronic Diabetic Neuropathy and Diabetes-Associated Cognitive Impairment Using Medicinal Herbs (*Cassia Angustifolia* and *Nigella Sativa*)

Mahum Khan[a,1], Hibba Riaz[a,1], Faria Hasan Jatala[a], Aneeqa Noor[a,*], Sara Mumtaz[b], and Saima Zafar[a,c]

[a]*School of Mechanical and Manufacturing Engineering (SMME), National University of Sciences and Technology (NUST), Islamabad, Pakistan;* [b]*Department of Biological Sciences, National University of Medical Sciences, Rawalpindi, Pakistan;* [c]*Clinical Department of Neurology, University Medical Center Göttingen and the German Center for Neurodegenerative Diseases (DZNE), Göttingen, Germany*

Nodal regions, areas of intensive contact between Schwann cells and axons, may be exceptionally vulnerable to diabetes-induced changes because they are exposed to and impacted by the metabolic implications of diabetes. Insulin receptors, glucose transporters, Na^+ and K^+ channels, and mitochondria are abundant in nodes, all of which have been linked to the development and progression of Diabetic Peripheral Neuropathy (DPN) and Type 1 Diabetes Mellitus (T1DM)-associated cognitive impairment. Our study aimed to evaluate if the administration of *Nigella sativa* (NS) and *Cassia angustifolia* (CA) prevented diabetes-associated nervous system deficits in hyperglycemic mice. We developed T1DM mice through Streptozotocin (STZ) injections and validated the elevations in blood glucose levels. NS and CA were administered immediately upon the induction of diabetes. Behavioral analysis, histopathological evaluations, and assessment of molecular biomarkers (NR2A, MPZ, NfL) were performed to assess neuropathy and cognitive impairment. Improvements in memory, myelin loss, and the expression of synaptic proteins, even with the retention of hyperglycemia, were evident in the mice who were given a dose of herbal products upon the detection of hyperglycemia. NS was more beneficial in preventing memory impairments, demyelination, and synaptic dysfunction. The findings indicate that including these herbs in the diets of diabetic as well as pre-diabetic patients can reduce complications associated with T1DM, notably diabetic peripheral neuropathy and cognitive deficits associated with T1DM.

*To whom all correspondence should be addressed: Dr. Aneeqa Noor, Email: aneeqa_n@yahoo.com; ORCID: 0000-0001-8692-3161.

Abbreviations: DPN, Diabetic peripheral neuropathy; T1DM, Type 1 Diabetes Mellitus; STZ, streptozotocin; MPZ, myelin protein zero; NfL, Neurofilament light chain protein; NS, *Nigella sativa*; CA, *Cassia angustifolia*.

Keywords: Streptozotocin, Diabetic Neuropathy, Cognitive Impairment, *Cassia Angustifolia*, *Nigella Sativa*

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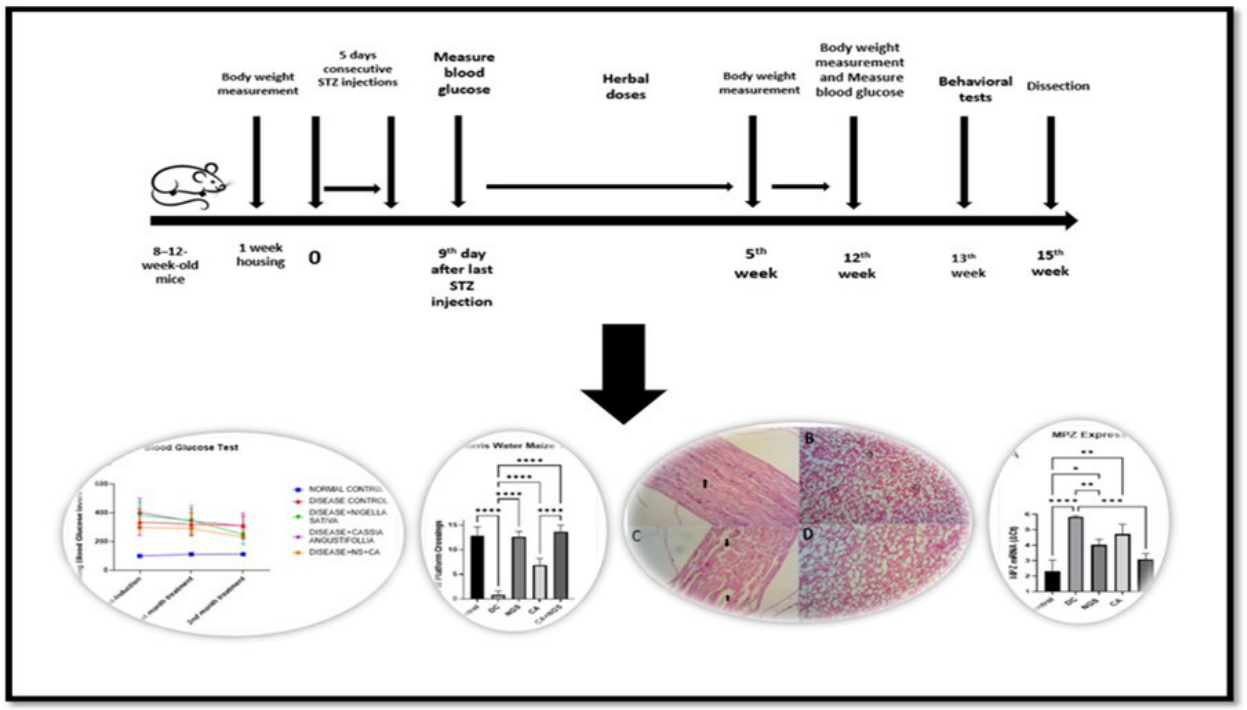
[1]Equal author contribution.



Graphical Abstract. **For the current study, STZ-induced models of Type 1 Diabetes Mellitus were generated**. The administration of herbs was started immediately upon the detection of hyperglycemia. The effect of the administration of herbs on neuropathy and cognitive impairment was evaluated through behavioral analysis, histopathology, and molecular evaluations.

INTRODUCTION

The prevalence of diabetes mellitus, a common yet potentially fatal medical disorder, has undergone a steady increase in the last few decades, thus making it a significant public health issue for the 21st century [1]. Diabetes mellitus has emerged as a colossal socioeconomic burden, particularly for impoverished nations, and has reached epidemic proportions worldwide. It is anticipated that the global incidence of diabetes will witness a 67% increase in emerging economies between 2010 and 2030 [2].

In the case of type 1 diabetes mellitus (T1DM), the pancreatic cells cease to produce insulin, primarily due to autoimmune damage. T1DM patients have a higher tendency to suffer from additional autoimmune disorders and mental health problems [3]. Complications typically associated with diabetes mellitus are commonly classified into two categories: microvascular and macrovascular disorders. The former includes diabetic kidney disease, retinopathy, and peripheral neuropathy, while the latter encompasses coronary heart disease, stroke, and peripheral arterial disease [4]. A rare form of peripheral nervous system neurodegeneration that mainly affects sensory, autonomic, and eventually, to a lesser degree, motor axons is known as diabetes-related neuropathy [5].

Diabetic peripheral neuropathy (DPN) is a frequent consequence of both type 1 and type 2 diabetes; it is a major contributor to neuropathic pain, which is incapacitating and often results in lower-limb amputations [6]. Recent studies have shed light on the cognitive effects of diabetes. Diabetes contributes to endothelial dysfunction and cognitive impairment, making it one of the risk factors for the onset of vascular dementia [7]. The etiology of DPN can be attributed to defective Schwann cells and nerve demyelination. The expression of myelin protein zero (MPZ), a structural protein that plays a crucial role in myelination, undergoes alterations during the progression of myelination. Novel markers of brain injury, such as Neurofilament light chain protein (NfL), along with myelin-specific circulatory mRNA (cmRNA), hold great potential as non-invasive methods for identifying early indications of DPN and predicting its onset. Therefore, the analysis of MPZ and NfL protein can prove to be instrumental in comprehending the structural changes underlying DPN and may even offer novel targets for therapeutic intervention [8]. On the other hand, N-methyl-D-aspartate (NMDA) receptor subunit 2A (NR2A) serves as a pivotal mediator of excitotoxicity and neuronal damage [9].

Extensive research is currently being conducted on

flavonoids, alkaloids, phenolic compounds, terpenoids, saponins, and phytosterol-like substances, which are present in herbal treatments utilized globally, to address diverse diabetes complications [10]. *Nigella sativa* (NS), commonly known as the fennel flower plant, has gained significant recognition [11]. Cassia species (Caesalpiniaceae) have recently sparked interest in botanical compounds and pharmacological studies due to their exceptional therapeutic properties. Their cathartic and purgative qualities have been extensively documented in traditional medicine [12]. The antioxidant assays of NS and *Cassia angustifolia* (CA) extracts, utilizing DPPH free radical scavenging at different concentrations, have exhibited their potential as antioxidants. Furthermore, these medicinal plants possess significant amounts of alkaloids, carboxylic acid, coumarins, phenols, resins, saponins, and steroids, signifying their potential as bio-constituents [13].

Herbs can interact with each other in ways that enhance their individual effects. This synergy can lead to greater efficacy than would be expected from the sum of the effects of each herb used separately [14]. Although CA extracts have been traditionally used for their laxative properties and have shown antibacterial activity in laboratory studies, they also have the potential to eliminate the debilitating implications of T1DM by controlling hyperglycemia [15]. On the other hand, NS has been studied for its potential neuroprotective properties, which may have implications for preventing or managing diabetic neuropathy. However, there is no research specifically exploring their combined effects for prevention [16]. Although these herbs have previously been studied for the treatment of complications associated with T1DM, the current study aimed to identify if their administration in the early stages of the disease can prevent the debilitating complications including DPN and cognitive impairment along with their synergistic effect.

MATERIAL AND METHODS

Ethical Approval

This study was approved by the Institutional Review Board, National University of Sciences and Technology (NUST), Islamabad, Pakistan.

Preparation of Herbal Extracts

NS oil (S. Amden group of companies Karachi, Pakistan), extracted from the seeds of the plant, was procured and utilized for this study. CA leaves were subjected to grinding and the resulting powder (100 g) was dissolved in 400 ml of distilled hot water over a period of 30 minutes. Following filtration with Whatman No 1 filter paper (1442 125, Cytiva, UK), the resulting complex was centrifuged at 3500 rpm for 20 minutes. The botanical extracts were then subjected to a hot water bath for a duration of 5 hours at 80°C. The purified extract was stored at a temperature of 5°C in a refrigerator until its utilization.

Development of Animal Models

Thirty Balb/c mice (n = 30) were procured from Animal House NUST and underwent a period of acclimatization lasting 1 week. Subsequently, their body weight was measured. On the inaugural day of disease induction, all food from each cage was removed 4 hours preceding STZ therapy by following the already optimized procedure [17]. The animals were then subjected to intraperitoneal administration of 40 mg/kg STZ or a continuous duration of 5 days. On day 14 ie., 9 days following the last STZ injection, all mice underwent a fasting period of 6 hours. Blood glucose levels from a tail vein sample were analyzed using a One Touch Basic blood glucose monitoring device to ensure hyperglycemia (above 150 mg/dl (8.3 mmol/L) in STZ-treated mice.

Following the induction of T1DM, the animals were treated with herbal dosages of NS and CA. An intragastric intubation of the liquid extract of CA at a dosage of 150 mg/kg body weight was administered for a total of 84 days. Additionally, NS was orally administered once daily at a dose of 400 mg/kg body weight. Body weight and glucose levels are measured again after 5 weeks and in the 12th week. Behavioral assessments were conducted during the 13th week, after which the animals were humanely euthanized and dissected for further PCR and histological testing.

Behavioral Tests

Eight behavioral tests were conducted by established protocols, four of which were relevant to diabetic neuropathy. Briefly for the hyperalgesia response, pain threshold was evaluated by paw licking or jump response in animals acclimated to a 50°C hot plate with a 60-second limit to prevent tissue damage [18]. For cold allodynia test, the animal's nociceptive response was assessed by counting the brisk lifting of its left hind paw on a cold metal plate (4 ± 1°C) over 5 minutes [19]. In paw pressure test, pressure was applied to mouse's paw using forceps, and the withdrawal threshold or latency was measured as an indication of pain sensitivity [20]. For the tail flick test, a heat source is focused on the animal's tail, and the latency to flick or withdraw the tail from the heat stimulus was measured [21]. The object location test assessed spatial memory retention in animals by allowing them to explore an environment with two identical objects, then moving one to a new location to measure recognition of the change [22]. For the Y-maze test, spontaneous alternation

Table 1. List of Primers Utilized in the Current Study. The forward and reverse primers for β-actin, NR2A, MPZ and NfL along with their optimized annealing temperatures have been stated.

Target	Forward Primer	Reverse Primer	Annealing temperature
β-actin	GCCTTCCTTCTTGGGTATGG	CAGCTCAGTAACAGTCCGC	61°C
NR2A (NMDA-receptor subunit 2A)	GAGACGGTCTTGGGATCTTA-AC	CCCATTCCCGGTCCTTATTC	49.9°C
MPZ (Myelin Protein Zero)	CCCTGGCCATTGTGGTTTAC	CCATTCACTGGACCAGAAG-GAG	62°C
NfL	GCGCCATGCAGGACACA	ACCTGGCCATCTCGCTCTT	61°C

behavior was evaluated as they freely explored the arms of the maze. The sequence and frequency of entries into each arm are recorded [23]. In the Morris water maze test, each mouse was given five acquisition trials per day for 5 consecutive days with a minimum inter-trial interval of 10 min and the average escape latency was calculated. On the 6th day, a spatial probe trial test was conducted where the platform was removed, and the animals were allowed to swim for 90 sec. We recorded the number of times the mice crossed the platform and along with the time spent in each quadrant [24]. In the open field test, the animals were placed in a novel open arena, and their spontaneous exploration, movement patterns, and time spent in specific zones were recorded to infer emotional responses and activity levels [25].

Histopathological Assessments

Hematoxylin and Eosin staining was performed on 5µ tissue sections and the slides were imaged using a Labomed microscope (Labo America Inc. USA). Image analysis was performed using ImageJ (version 1.53) software.

In Silico Analysis and Quantitative Polymerase Chain Reaction (qPCR)

Molecular docking using *in silico* analysis was done via Vina Wizard module inbuilt in PyRx and the results were obtained for all the proteins (PDB accession numbers 21971831 for MPZ (3OAI), 22723690 FOR NfL (NEFL), 16281028 for Nr2a (2A5S)) and ligands (thymoquinone, carvacrol, t-anethole, 4-terpineol from NS and Quercimeritrin, Rutin, scutellarein from CA). Analysis of the interactions of Protein-Ligand complex was done in BIOVIA Discovery Studio for a 3D visualization and a 2D image depicting bonding between the protein and ligand. The complex was then submitted to PyMOL for producing a protein-ligand complex and then subjected for a 2D image and a detailed bonding interaction in the complex.

Brain and sciatic nerve tissue were obtained from anesthetized mice following neck dislocation. RNA extraction and cDNA synthesis were performed. qPCR was conducted using the primers summarized in Table 1. The thermocycling settings were 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 30 s at 95°C, 1 minute at (61°C for beta-actin and NfL, 62°C for MPZ, 49.9°C for Nr2a), 1 minute at 72°C, and then a final dissociation step. The expression of all targets was normalized to the expression of β-actin and the data were analyzed using $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

Data visualizations and statistical analysis were conducted using GraphPad Prism version 10.0.0 (for Windows, GraphPad Software, Boston, Massachusetts USA). All analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test and the p-value was considered significant.

RESULTS

Biochemical Analysis

The arterial blood sugar levels of the animals were demonstrated. The diabetic mice showed insulin deficiency and hyperglycemia throughout the study. The initial weight of the mice was similar across all the groups during the trial. The diabetic animals lost weight after the treatment, whereas the control mice, NS, CA, and combination diabetic animals did not demonstrate any difference in their baseline and final body weights (Figure 1).

Behavioral Tests

Assessment of Neuropathy: The nociceptive threshold in normal mice was found to be notably influenced by NS, CA, and CA+NS. In comparison to healthy mice, diabetic mice exhibited marked reductions in threshold for pain latency. However, these reductions were significantly and dose-dependently reversed by NS, CA, and CA+NS. Furthermore, when mice were subjected to the administration of NS or CA+NS, the overall count of paw lifts was notably diminished in contrast to mice that were administered STZ as a control. In comparison to the disease control group of mice, the frequency of paw lifts in

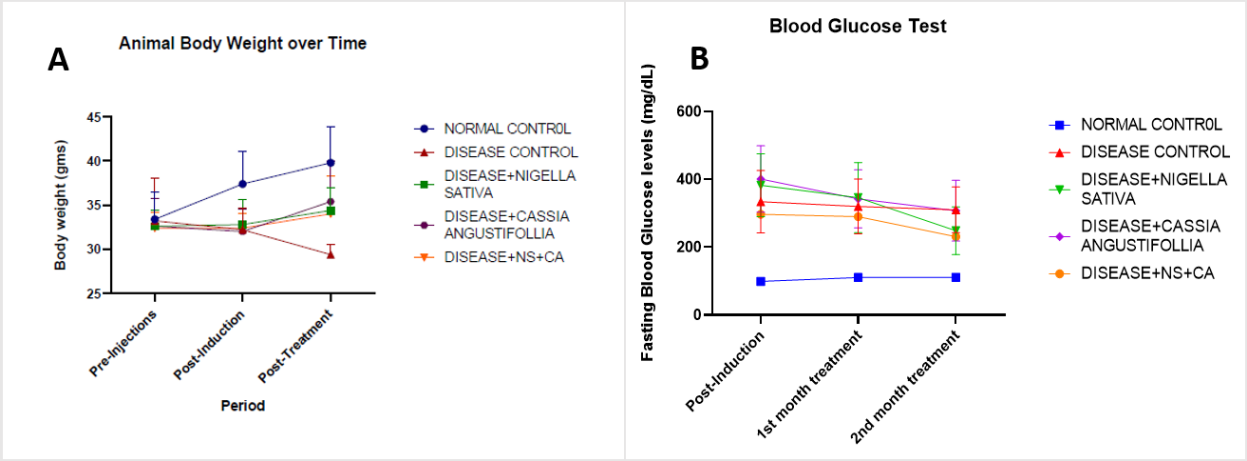


Figure 1. **Animal body weight and blood glucose levels, pre- and post-induction of disease**.

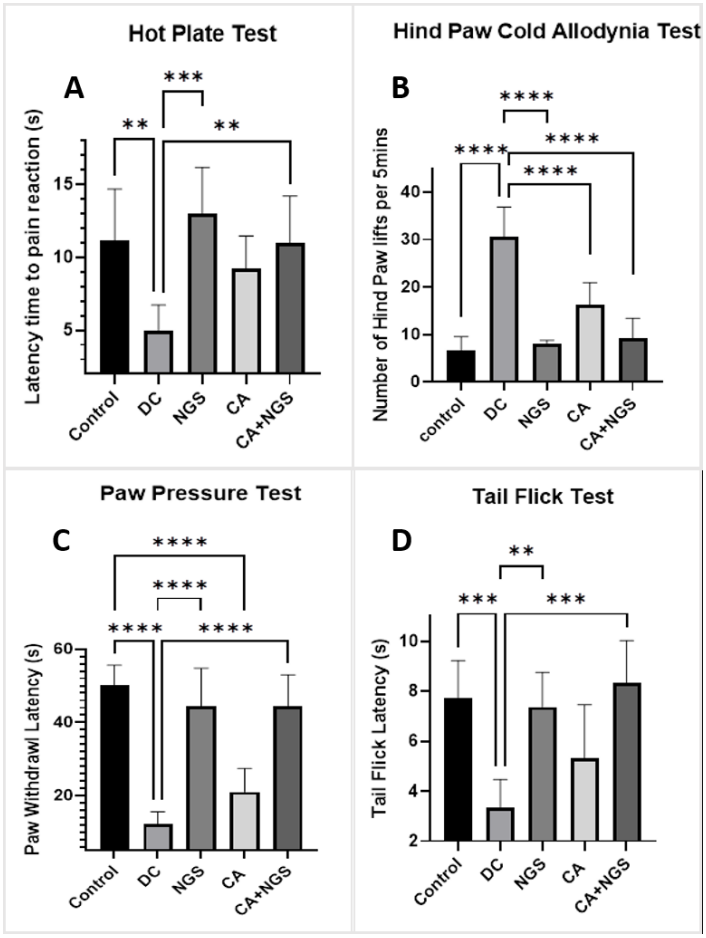


Figure 2. Diabetic neuropathy test: (**A**) When juxtaposed with the cohort of disease controls, the outcomes of the hot plate hyperalgesia examination demonstrated augmented hot-plate latencies. (**B**) When contrasting with the control group of diabetics, the cold allodynia assessment disclosed a diminished quantity of paw elevations. (**C**) The paw pressure test experiment has produced results indicating a significant increase in the paw withdrawal threshold of the control group with the disease. (**D**) The Tail Flick test has demonstrated that the tail withdrawal latency in the treatment groups was greater than that of the control group with the disease. The statistics are presented as mean (SD) for each of the five groups: *p = 0.05, ** p = 0.01, *** p = 0.001, **** p = 0.0001 by one-way ANOVA with Tukey multiple comparisons test in all (**A**, **B**, **C**, and **D**).

CA-treated mice underwent a slight augmentation, albeit not statistically significant, in the aftermath of treatment. Additionally, the latency of the pain threshold experienced by diabetic mice was significantly decreased in comparison to that of normal and healthy animals. However, this reduction was greatly mitigated in a dose-dependent manner by the administration of NS, CA, and CA+NS. Lastly, significant elevations in tail flick latencies were observed in both NS and CA+NS treated animals when compared to the STZ control group of mice. Additionally, post-treatment, CA-treated mice exhibited a minor, albeit non-significant, reduction in tail flick latency relative to the disease control (Figure 2).

Assessment of Cognitive Impairment: The experimental findings indicate that mice with diabetes exhibited markedly reduced exploration time relative to their non-diabetic counterparts in object location tests and the administration of herbs reverted the detrimental effects of diabetes. Notably, no meaningful distinction in exploration time was detected between mice treated with NS and those administered with extracts of the two herbs. Additionally, the findings of the Y-maze experiment evinced an absence of noteworthy contrast in the frequency of entries made by diabetic mice in the novel arm in comparison to the other arm. Conversely, the treated mice exhibited a greater proclivity towards entering the novel arm as opposed to the other arm. Moreover, the mice that were administered both extracts demonstrated a penchant for entering the novel arm over the other arm, as depicted in Figure 3.

Furthermore, the Morris Water Maze test was employed to evaluate deficits in spatial memory. During the 6th day of the probe test, it was observed that mice injected with STZ exhibited a reduced number of entries and crossing time in the target quadrant in comparison to those that were not injected. Additionally, diabetic mice displayed a lower number of platform crossings as opposed to normal mice. Conversely, treated mice did not exhibit any significant deviations in the time spent or platform crossings in comparison to their normal counterparts. The group of mice treated with both extracts displayed the highest number of entries. It was found that the NS group did not exhibit any significant divergence as compared to the control group. The number of entries of mice treated with CA fell in between the disease and control groups as illustrated in Figure 3. Similarly, the findings of the open field test unambiguously demonstrate that mice afflicted with diabetes were constrained to the periphery and exhibited a markedly diminished frequency of entries in the central area. Conversely, the mice treated with NS and CA demonstrated a proclivity to investigate the central area. Moreover, the cohort that administered both extracts displayed the highest amount of time spent in the central area, as depicted in the accompanying Figure 3.

Histopathological Analysis

Histological examination of the sciatic nerves in STZ-induced diabetic mice demonstrated a myelin coating of the myelinated nerve fibers that were weak, loose, and disordered. Upon administration of NS, CA, and combined CA+NS extracts, the preventative and curative groups exhibited only minor degradation of the myelin sheath. There was no visible injury in the control category. The experimental groups underwent computer-aided morphometric analyses of myelinated nerve fibers. Similarly, in the case of brain samples, neuronal loss was prevented upon the administration of herbal doses and NS showed the most profound effects (Figure 4).

Relative Expression of Biomarkers

The molecular evaluation was performed using NR2A, MPZ, and NfL. Their binding with the active ingredients of compounds NS and CA was evaluated through molecular docking. Quercimeritin and Carvacrol showed the highest average binding energies with our targets among the active ingredients of CA and NS respectively (Figure 5). Subsequently, data generated from qPCR was utilized for evaluating the expression of MPZ, NfL, and NR2A proteins. In comparison to the diseased groups, there was an increase in the expression of MPZ in the preventive groups, while NfL expression was downregulated in the prevention groups, similar to the levels observed in healthy controls. Additionally, NR2A expression also reverted to healthy levels upon the administration of NS.

An evaluation of the data generated from the quantitative polymerase chain reaction experiment is necessary to analyze the substantial variations in the expression of MPZ, NfL, and Nr2a proteins between the control and diseased mice. The treated groups exhibited an improvement in the expression of proteins, and the extent of protein expression up- and down-regulation in various groups was evident. In comparison to the diseased groups, there was an increase in the expression of MPZ in the preventive groups, while NfL expression was downregulated in the prevention groups (Figure 6). However, the delta-delta CT graph demonstrated a 0.5-fold relative expression to the housekeeping gene beta-actin for -1 and a 2-fold relative expression for +1.

DISCUSSION

Diabetes mellitus, commonly known as diabetes, comprises a range of disorders with autoimmune, metabolic, and genetic origins that cause hyperglycemia [26]. The main precipitating factor for diabetic neuropathy is prolonged exposure to elevated levels of blood glucose, characterized by neuronal damage and disruption of

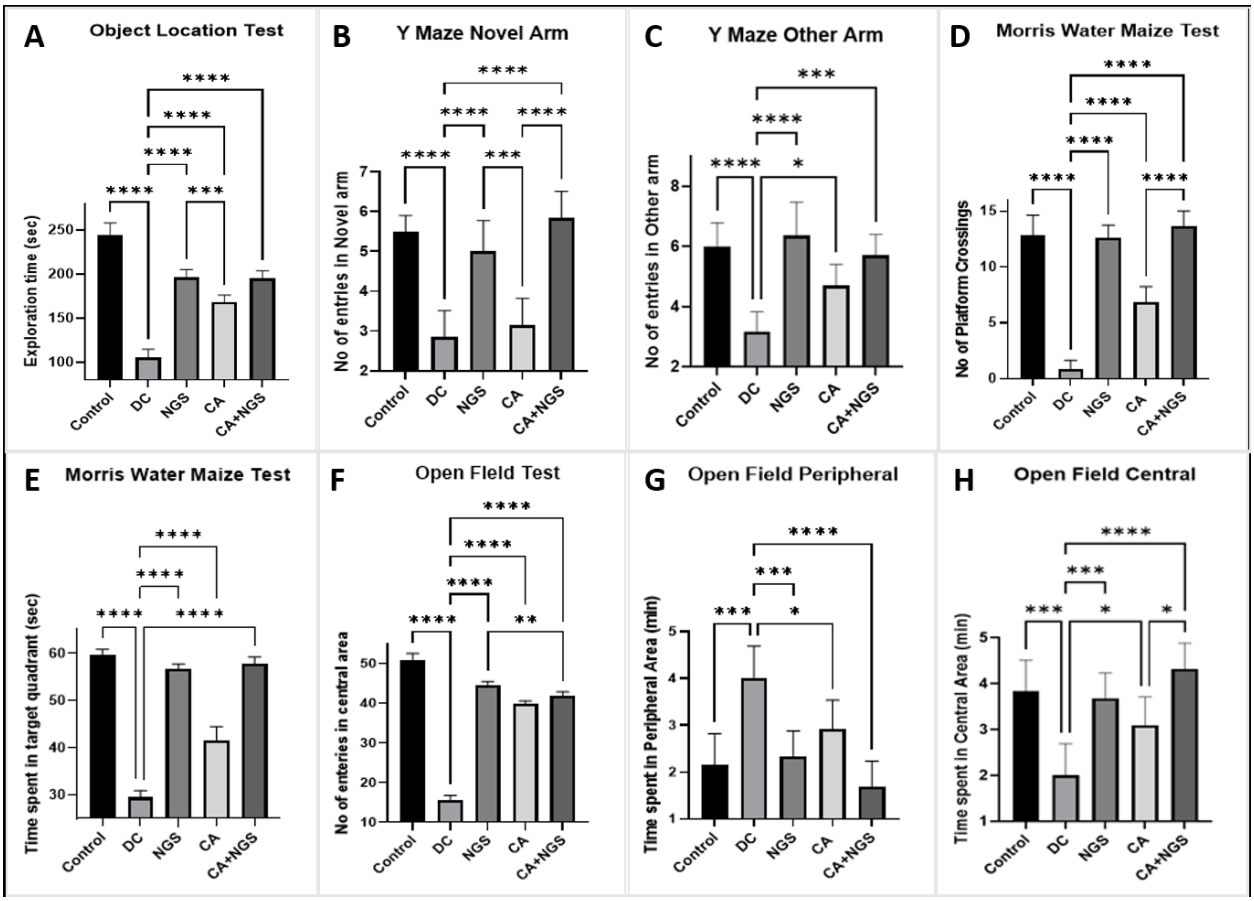


Figure 3. **Cognitive Impairment Tests**: (**A**) Mice afflicted with the disease spent the least amount of time in their exploration of the novel object. (**B**&**C**) quantity of entries of mice that were subjected to the extract in the novel arm exhibits remarkable enhancement among all groups. (**D**) mice subjected to NS and CA treatments exhibited the highest frequency of entries in the target quadrant. (**E**) The experimental mice that received both extracts exhibited the highest quantity of platform crossings. (**F**&**G**) cohort administered with both extracts exhibited a substantial duration in the designated quadrant of interest. (**H**) mice treated with a combination of both herbs spent the greatest amount of time in the central area. The one-way ANOVA method, followed by Tukey's multiple comparison test was used and all (**A**, **B**, **C**, **D**, **E**, and **F**) showed significant results.

nerve signaling [27]. In this study, we investigated the potential beneficial preventive effects of NS, CA, and their combined effect on DPN in STZ-induced diabetic mice. A significant amount of work has been directed toward the role of oxidative stress as a crucial pathophysiological mechanism in the development of DPN. The overproduction of reactive oxygen species and the disruption of antioxidant defense systems lead to a disturbance in redox balance, thereby promoting oxidative damage in DPN. These findings provide innovative therapeutic approaches for DPN that target oxidative stress. At week 12, rats with STZ-induced diabetes exhibit symptoms of diabetes and diabetic complications, including peripheral sensory nerve injury [28].

Previous research has established that commonly used plant chemicals possess advantageous neuroprotective, antioxidative, and anti-neuroinflammatory properties that can effectively enhance DPN. Our research has focused on the significance of oxidative stress in the pathogenesis of DPN, as our *in silico* analysis demonstrated that all the antioxidant compounds from both herbs CA (quercimeritrin, scutellarein, rutin) and NS (thymoquinone, carvacrol, t-anethole, 4-terpineol) exhibit strong binding energies and mostly favorable interaction with structures of both protein MPZ and NfL involved in the pathology of DPN, as well as with the structure of protein Nr2a, which is involved in the pathology of cognitive impairment. Previous studies have indicated that patients with diabetic neuropathy have a deficient endogenous opioid peptide system that is linked with heightened pain perception [29-31].

Our analysis of hyperalgesia and allodynia in mice revealed a significant disparity between control and diseased subjects. However, herbal doses were found to

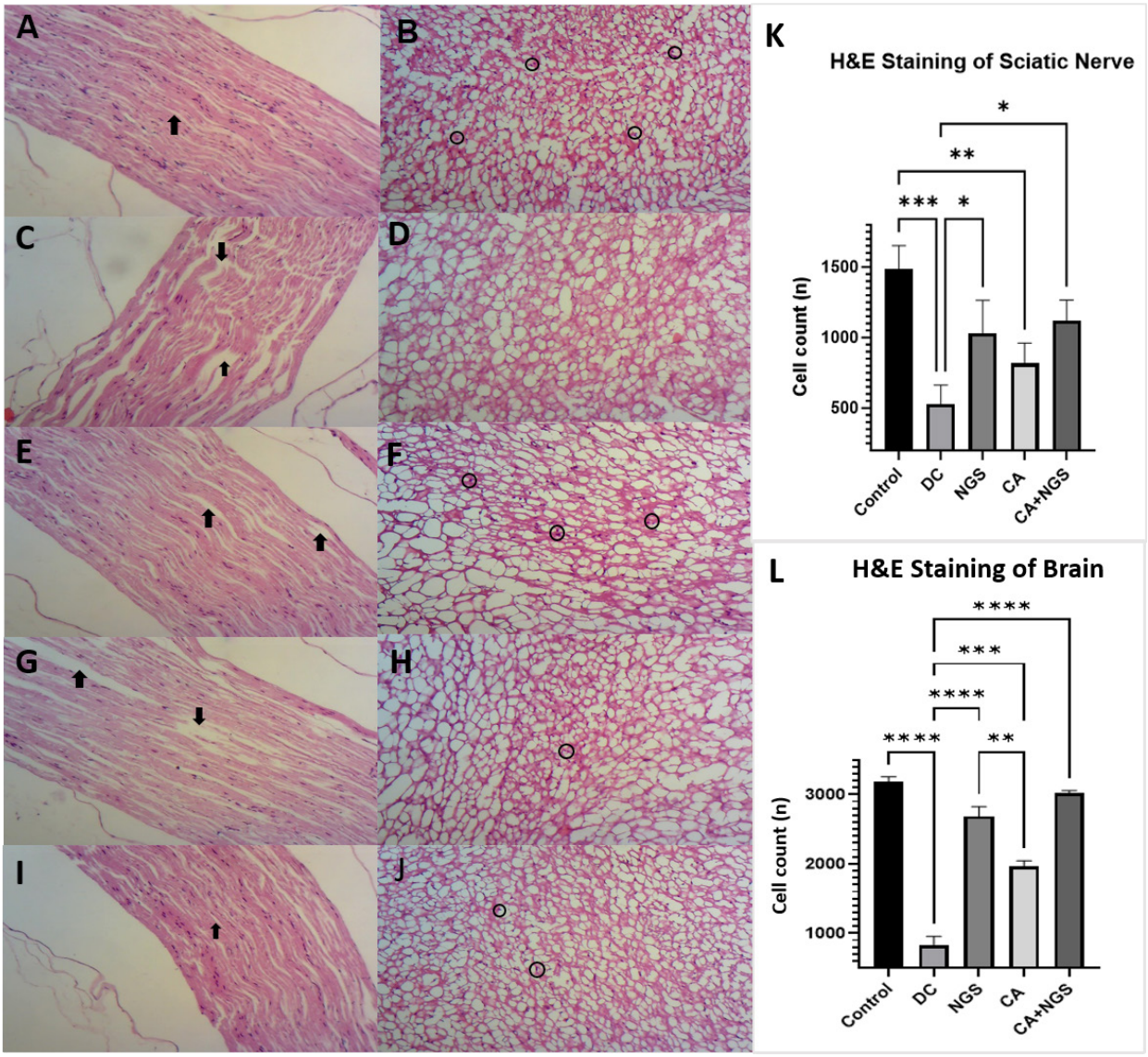


Figure 4. **Sciatic Nerve and brain tissues stained with Hematoxylin and Eosin**. (**A**) Sciatic nerve of mice of the control group. (**B**) Brain tissues of mice of the control group. (**C**) Sciatic nerve of diabetic mice. (**D**) Brain tissues of diabetic mice. (**E**) Sciatic nerve of mice treated with NS. (**F**) Brain tissues of mice treated with NS. (**G**) Sciatic nerve of mice treated with CA. (**H**) Brain tissues of mice treated with CA. (**I**) Sciatic nerve of mice treated with both CA & NS. (**J**) Brain tissues of mice treated with both CA & NS. Arrow heads shows demylination of nerve fibers in sciatic nerve while circles shows degeneration of neurons in brain's cortex. (**K**) Graph showing Schwann cell nuclei count from histopathology analysis of sciatic nerve. (**L**) Graph showing neuronal count from histopathology analysis of brain tissue. The statistics are presented as mean (SD) for each of the five groups: * p = 0.05, ** p = 0.01, *** p = 0.001, **** p = 0.0001 by one-way ANOVA with Tukey multiple comparisons test.

improve the DPN pathology. The mechanisms underlying both demyelinating neuropathy and axonopathy suggest that the impairment or dysregulation of Schwann cells in the sciatic nerve is involved [32]. H&E staining of sciatic nerve tissue from DPN mice indicated demyelination and dysregulated Schwann cells, while treated groups displayed improvement. Furthermore, the brain exhibited a decrease in neuronal count in the case of DPN, which was ameliorated with herbal treatment. Previous studies have noted that participants with diabetic neuropathy exhibit a significant decrease in mRNA myelin protein zero expression levels, while NfL protein levels are increased [8]. Similarly, our q-PCR data showed a decrease in MPZ mRNA expression in a mouse model of DPN, while treat-

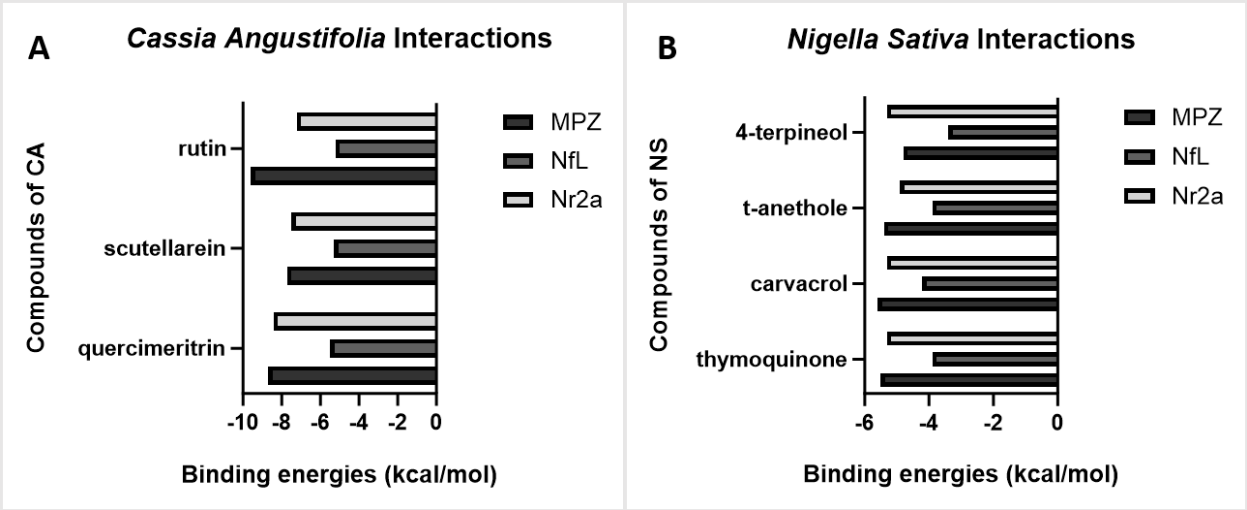


Figure 5. ***In silico*** **analysis to reveal the interaction of active compounds with relevant proteins**: (**A**) Binding energy graph of MPZ, NfL and Nr2a with compounds of CA. (**B**) Binding energy graph of MPZ, NfL, and Nr2a with compounds of with compounds of NS. Binding energies evaluated by PyRx are shown in the graph. Rutin is shown having the highest binding energy at -9.6 kcal/mol and in case of compounds of NS, carvacrol shown to have the highest binding energy of -5.6 kcal/mol.

ment with NS, CA, and a combination of the two led to a relative increase in mRNA expression. The observed upregulation of MPZ suggests a restoration of myelin integrity, which is crucial for maintaining nerve function and preventing neurodegeneration [33]. Similarly, the downregulation of NfL indicates a reduction in neuronal damage and neuroinflammation, further supporting the neuroprotective effects of herbal treatment [34]. Moreover, the restoration of NR2A expression to healthy levels suggests a normalization of synaptic function, which may contribute to the observed cognitive enhancement following herbal treatment [35].

On the other hand, NfL mRNA expression in the DPN mouse model increased, while herbal treatment groups showed a decrease in its expression. The hippocampus and prefrontal cortex manifest a reduced expression of synaptic proteins, including the NMDAR subunit NR2A. These observations imply that there are similarities in the alteration patterns of synaptic proteins in STZ-induced T1DM mice that may reveal mechanisms underlying T1DM-induced cognitive impairment [36]. Our PCR results corroborate the above study, as there was a significant decrease in the expression of Nr2a in diseased mice samples, while mice treated with herbs showed an improvement in Nr2A mRNA expression. The sciatic nerves that were exposed to STZ exhibited a more favorable response in both biochemical and morphological aspects when treated with NS therapy. Research indicates that the direct and indirect antioxidant activities of NS in the treatment of DPN are the determining factors responsible for its neuroprotective properties [37].

Previously, a study was conducted that demonstrated that the administration of NS extract and thymoquinone to rats with cerebral hypoperfusion led to enhancements in their learning and memory processes by reducing hippocampal oxidative stress and AChE activity [38]. The neuroprotective activity of CA extract is linked to its antioxidant properties, which serve to decrease oxidative stress on susceptible neurons, leading to improved neuronal function and reduced neuronal damage. Dementia is characterized by neural circuit degeneration, reduced levels of brain acetylcholine, and impaired neuronal transmission [39]. Our histological examination revealed structural alterations in the sciatic nerves and brain tissues of diabetic mice, characterized by myelin degradation and neuronal loss, respectively. Remarkably, treatment with NS, CA, and their combination preserved the integrity of the myelin sheath and prevented neuronal loss, highlighting their protective effects against diabetes-induced neuropathy and neurodegeneration. These findings support the notion that herbal interventions may exert neuroprotective effects through mechanisms involving myelin maintenance and neuronal survival [40].

Overall, these findings provide compelling evidence for the molecular mechanisms underlying the therapeutic effects of herbal interventions in mitigating neuropathy and cognitive impairment associated with diabetes. Our study provides compelling evidence for the therapeutic potential of NS, CA, and their combination in ameliorating biochemical, behavioral, histopathological, and molecular abnormalities associated with diabetes in mice. These herbal interventions exerted multifaceted

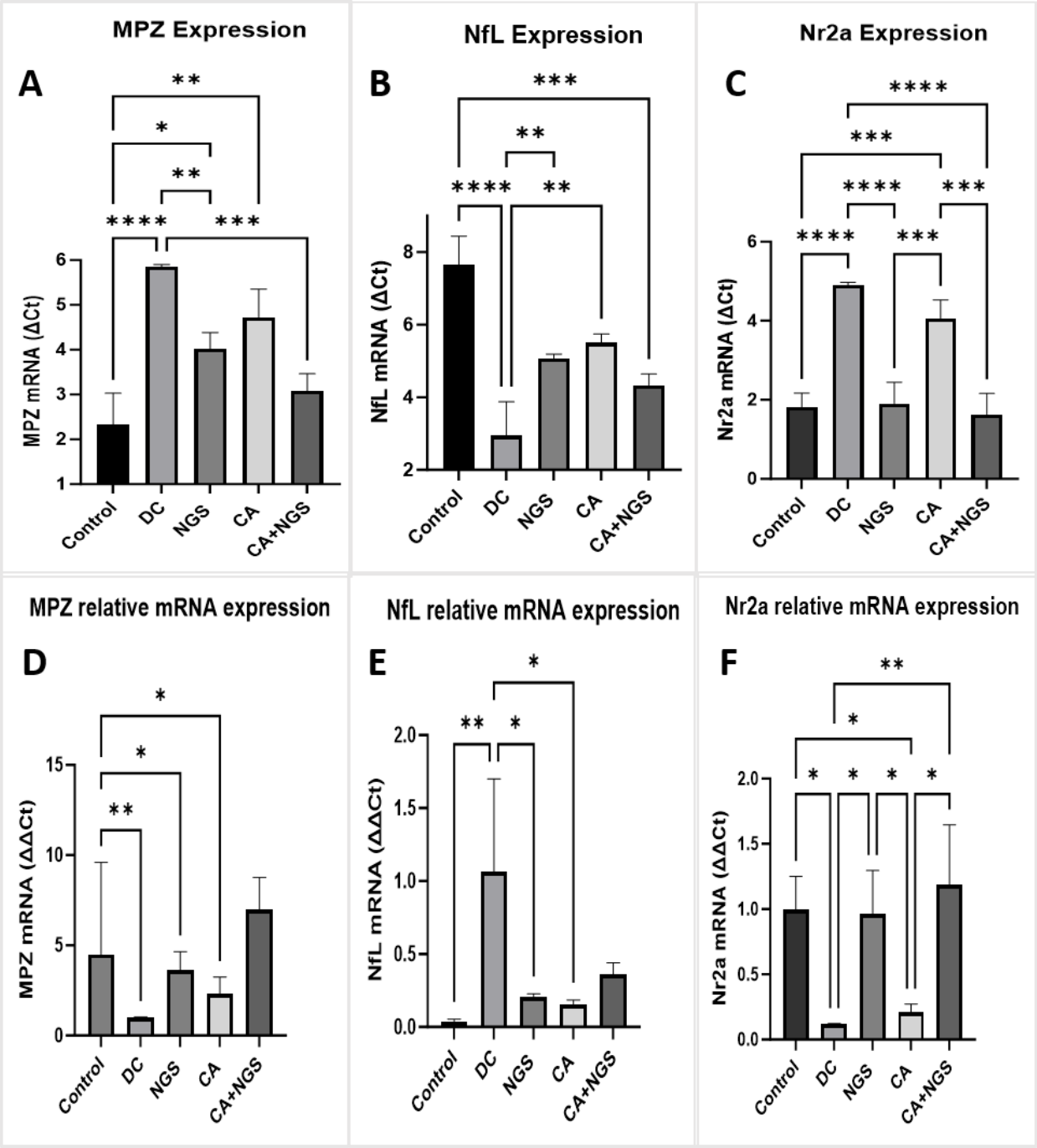


Figure 6. **ΔΔCt values after qPCR Analysis**. CT values from both replicates were used to calculate the expression of proteins and were normalized by β-actin and relative to the different experimental groups ΔCt values are calculated and are shown in graphs. A significant disparity in protein expression of (**A**) MPZ, (**B**) NfL and (**C**) Nr2a was observed between control and diseased mice. Using the ΔΔCT method the relative mRNA expression of (**D**) MPZ, (**E**) NfL, and (**F**) Nr2a are shown. A significant difference in both MPZ and NfL protein expression among the control and diseased mice was observed. The treated groups exhibited an amelioration in protein expression, and the level of up-regulation and down-regulation in the expression of both proteins in different groups is apparent. Encouragingly, treated groups showed an improvement in protein expression. One-way ANOVA with Tukey's multiple comparisons test was performed for statistical analysis. The error bars represent the standard deviation.

effects, including metabolic regulation, neuroprotection, and cognitive enhancement, highlighting their promise as novel therapeutic strategies for managing diabetes and its complications. Further translational research is warranted to elucidate the underlying mechanisms of action and to evaluate the safety and efficacy of these herbal treatments in clinical settings.

CONCLUSION

We worked on cognitive impairments associated with T1DM in mouse models and obtained promising results by treating T1DM mice by natural herbs. There is hope of a cure for diabetic neuropathy in these herbal medicines. Moreover, herbal medicines have less side effects as compared to traditional allopathic medicines. Our findings support the notion that NS and CA possess neuroprotective and antioxidative properties, mitigating DPN pathology. The preventive efficacy of CA and NS in chronic diabetic neuropathy and diabetes-associated cognitive impairment holds significant real-world importance, promising to substantially enhance the quality of life for individuals with diabetes while potentially reducing healthcare costs globally. Offering alternative treatment options aligned with natural and holistic preferences, these findings could alleviate the burden of diabetes-related complications, especially in regions with limited healthcare access. The potential synergistic effects of these herbs for preventing diabetic neuropathy also holds significant promise for both clinical practice and public health initiatives. This combination could offer a novel and accessible treatment option for individuals with diabetic neuropathy, potentially providing relief from symptoms and slowing disease progression. Moreover, it can stimulate further exploration of herbal remedies, fostering the discovery of additional natural treatments and advancing herbal medicine research, ultimately benefiting diabetic populations worldwide.

Conflict of interest: The authors declare that no conflict of interest is associated with this study. Prior to or following the research project's start, none of the authors had any actual or potential conflicts regarding interests that might have improperly influenced their work. The authors of this paper have done this work impartially and objectively because they are dedicated to sustaining the highest level of scientific and academic quality.

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