Effects of *Cassia Angustifolia* and *Nigella Sativa* for the prevention of Diabetic Neuropathy

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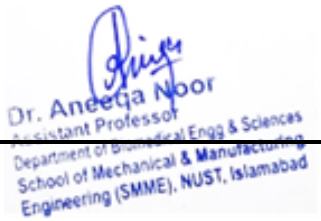

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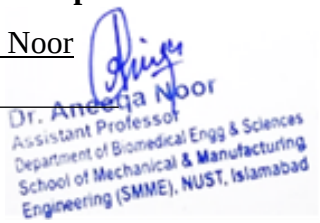

Copyright Statement

Dedication

*I DEDICATE THE OUTCOME OF MY EFFORTS TO MY PARENTS MR. AND MRS. PERVEZ SALEEM AND MY BELOVED MAMU MR. SARDAR SHOAIB WHO HAVE ALWAYS STOOD BY MY SIDE AND SUPPORTED EVERY DECISION I'VE EVER MADE***.**

Table of Contents

List of Figures

List of Tables

List of Abbreviations

DM	Diabetes mellitus
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
DPN	Diabetic Peripheral Neuropathy
CSF	Cerebrospinal Fluid
STZ	Streptozotocin
DN	Diabetic Neuropathy
RNS	Reactive Nitrogen Oxidative Species
ROS	Reactive Oxidative Species
AGEs	Advanced Glycated end Products
RAGE	Receptor for Advanced Glycated end Products
MODY	maturity-onset of the young
PKC	Protein Kinase C
IL-6	Interleukins-6
TNF	Tumor Necrosis Factor-alpha
EAHM	East Asian Herbal Medicine
WHO	World Health Organization
NIDDM	Non-Insulin-Dependent Diabetes mellitus
SOD	Superoxide Dismutase
CA	*Cassia angustifolia*
NGS	*Nigella sativa*
cDNA	Complementary DNA
qPCR	Quantitative Real Time Polymerase Chain Reaction
SEM	standard error of the mean
PMP22	peripheral myelin protein 22

PLP1	proteolipid protein 1
AD	Alzheimer's Disease
HIV	Human immuno-deficiency virus
HNF	Hepatocyte nuclear factors
PAD	peripheral artery disease
MBP	Myelin basic protein
NfL	neurofilament light chain
MPZ	myelin protein zero

Abstract

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). We gave natural herbs i.e., *Nigella sativa* and *Cassia angustifolia* to hyperglycemic mice after STZ injections to check their potential in preventing DPN. We saw improvements in demyelination, neuronal loss, and oxidative stress despite sustained hyperglycemia. We investigated the expression of MPZ and NFL in sciatic nerve and brain tissue samples from STZ-induced Type 1 Diabetes Mellitus (T1DM) mice models and compared control and dose-administered groups to diseased groups. Statistical analysis revealed a significant difference between diabetic mice and mice who were given herbs upon the induction of disease. 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.

Chapter 1
Introduction

1.1. Diabetes

Diabetes, also referred to as Diabetes Mellitus (DM), is an umbrella term of autoimmune in origin, metabolic, and genetic diseases characterized by hyperglycemia. Diabetes is a persistent medical ailment that hampers the body's capacity to control blood glucose levels. This condition occurs when the pancreas fails to produce a sufficient quantity of insulin, leading to Type 1 Diabetes Mellitus (T1DM). Additionally, it can also result from the body developing resistance to the effects of insulin, which characterizes Type 2 Diabetes Mellitus (T2DM). Insulin is a hormone responsible for facilitating the transfer of glucose (sugar) from the bloodstream to cells, where it can be utilized for energy. When insulin is insufficient or ineffective, blood sugar levels rise, creating a number of health problems (Bjelakovic et al., 2009).

Numerous episodes of urine retention, ongoing thirst, unanticipated weight loss, weariness, hazy vision, and slow wound healing are all signs of diabetes. If diabetes is not adequately controlled, this condition can give rise to significant complications, including heart disease, nerve damage, kidney impairment, and vision issues.T1DM typically develops during puberty or infancy and requires lifelong insulin therapy. A healthy diet, regular exercise, and medications can sometimes be used to treat T2DM, which is more prevalent and frequently brought on by lifestyle factors. However, some T2DM might need to use insulin or other medications to control their blood sugar levels (Cloete L, 2022).

Monitoring the glucose levels, eating a healthy diet, exercising, taking prescribed medications, and scheduling routine appointments with medical professionals are all part of managing diabetes. Diabetes support and education are essential for helping diabetics' live healthy lives. T1DM presents few opportunities for early identification, however T2DM is now well supported by research. With proper care and self-management, secondary prevention is possible in both types of diabetes. To create evidence-based, culturally appropriate diabetes treatment recommendations, and T2DM management requires an accurate mechanism that not only gathers local data but also evaluates and tracks the situation. Lack of national monitoring and diabetes research is a major barrier

to addressing the T2DM crisis in Pakistan. Many international organizations and local professionals have raised similar concerns (Vlachou et al., 2022).

1.1.1. Types of Diabetes

Diabetes of the first kind T1DM, diabetes of the second kind T2DM, additional particular kinds of diabetes, and pregnancy-related diabetes are the four main subtypes of DM. The elimination of pancreatic β-cells, which is typically brought on by autoimmune inflammatory processes, is a hallmark of T1DM. Serum autoimmune markers encompass islet cell autoantibodies, autoantibodies targeting the enzyme glutamic acid decarboxylase (GAD), insulin, tyrosine phosphatases IA-2 and IA-2b, as well as the zinc transporter ZnT8. While the pace of progression may differ, this detrimental process commonly culminates in total insulin deficiency and unnoticeable plasma C-peptide levels (DeFronzo et al., 2015).

Complex metabolic disease diabetes type 2 is characterized by β-cell dysfunction and different levels of resistance to insulin can also be observed. Other metabolic conditions that can coexist with T2DM include obesity, hypertension, and polycystic ovarian syndrome. This resistance to the hormone insulin is also common in these conditions. This is defined as having a commencement or first discovery of overt pre-gestational diabetes while pregnant, excluding women who are likely to have the condition. Medical conditions related to pregnancy often lead to an increase in adverse outcomes. Although these issues typically resolve following childbirth, individuals affected should undergo postnatal diabetes screening and be informed about their elevated risk of developing T2DM in the future (Joshua et al., 2022).

T2DM has an unclear genetic basis, whereas many other types have a known genetic basis. Diabetes with maturity-onset of the young, a family variant of the disease brought on by mutations in β-cell or hepatic genes (such as glucokinase and HNF homeobox A (HNF-1α), is the most prevalent of these. Other well-known types of diabetes, also known as secondary diabetes, include those brought on by pancreatic disease (such as hemochromatosis-related diabetes), excessive corticosteroid hormone, or specific

medications (such as protease inhibitors in HIV infection, unusual immune spot check inhibitors in cancer treatment and antipsychotics in schizophrenia,) (Liu et al., 2023).

1.1.2. Prevalence of DM

Pakistan has a high prevalence of diabetes, ranging from 7.6 to 11%. There are variations according to age, gender, location, and urbanization. In terms of frequency and relative prevalence of DM in various regions and among women and men, the results of the National Diabetes Survey along with the National Health Survey are different. The annual rate of Type 1 Diabetes Mellitus (T1DM) in Karachi, Pakistan, was estimated at 1.02 per 100,000. In Pakistan, the prevalence of gestational diabetes falls within the range of 3.2% to 3.5%, as compared to other countries. Nevertheless, rates of complications for both the child and the mother were observed to be higher, likely due to suboptimal glycemic control (Im et al., 2022).

1.1.3. Complications related to DM

Patients with T1DM or T2DM diabetes frequently experience diabetes-related complications, which also significantly increase morbidity and death. Diabetes gives rise to chronic complications, approximately characterized into macrovascular and microvascular, with the past existence notably more predominant. Microvascular complications include retinopathy, neuropathy, and nephropathy, while macrovascular complications involve stroke, peripheral artery disease, and cardiovascular disease. Diabetes stands as a primary provider to lower limb amputation, with diabetes-related foot syndrome characterized by the presence of a neuropathy, coupled with foot ulcer, infection, and peripheral vascular dysfunction. Additionally, there are other diabetic complications not covered in the previous groups, such as dental issues, diminished infection resistance, and challenges during childbirth in women with gestational diabetes (Papatheodorou et al., 2018).

Initial reversible metabolic changes are gradually replaced by structural degenerative changes, which ultimately result in the loss of nerve fibers. T1DM, in contrast, is more significantly impacted by the late structural stages of Diabetic Peripheral Neuropathy (DPN). Progressive axonal shrinking and loss are more pronounced in T1DM.

Additionally, T1DM DPN differs from T2DM DPN in that it has para nodal degenerative changes. In T1DM, defects in insulin action and transmission of signals can be blamed for these disparities because they affect how neurotrophic substances, and their receptors are expressed. Following effects on neuroskeletal and sticky protein molecules, their posttranslational modifications, and nociceptive peptides result in the more severe pathophysiology in T1DM DPN. Future interventional approaches to treat these prevalent diseases should take into account these variations in underlying mechanisms.

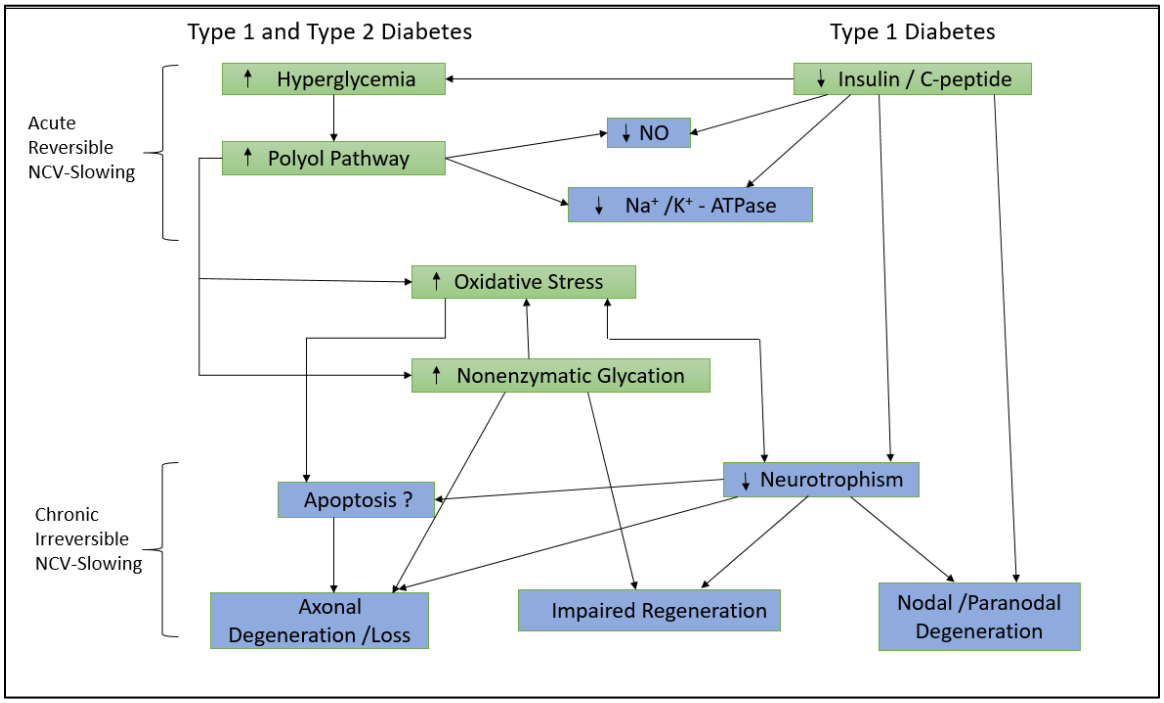

Figure 1: Pathogenesis pathways implicated in T1DM and T2DM DPN. The initial metabolic imbalances that underpin acute functional impairments are reversible. These are gradually piled by escalating structural anomalies, which became less reversible following metabolic adjustments.

1.2. Diabetic Neuropathy (DN)

An epidemic of the repercussions of these illnesses has resulted from the worldwide epidemic of diabetes and prediabetes. The most frequent side effect is neuropathy, with distal symmetric polyneuropathy (also known as diabetic neuropathy) being one of its most common manifestations. DN is a sensory function deficit that starts distantly in the lower limbs and is accompanied by severe morbidity and discomfort.

A form of nerve damage that occurs in diabetics, particularly in those with poorly controlled blood sugar levels, is diabetic neuropathy. It is a frequent side effect of diabetes that can leads to many symptoms, which includes pain, tingling, and numbness in the fingers, toes, and other parts of the body (Vinik et al., 2013). Long-term exposure to high blood glucose levels harms neurons and interferes with nerve communication, which results in DN (Callaghan et al., 2012). Oxidative stress, inflammatory conditions, and mitochondrial dysfunction are some of the complex interactions that lead to DN (Pop-Busui et al., 2017). Individuals with T2DM are more likely to develop diabetic neuropathy than individuals with T1DM.

According to a meta-analysis of 99 studies with over 400,000 patients that were published between 2007 and 2017, the incidence of DN is thought to be 28.5% in people with T1DM and 48.9% in those with diabetes who have T2DM (Tesfayeet al., 2011). Treatment options for DN include dietary adjustments, prescription medicines, and surgical nerve decompression treatments (Jeffcoate et al., 2018). According to a 2018 randomized controlled trial published in the journal The Lancet Diabetes & Endocrinology, the drug tapentadol is effective at easing the pain related to DN (Baron et al., 2017).

1.2.1. Diabetic peripheral neuropathy (DPN)

DPN is widespread, impacting 40% to 50% of the total diabetic patients (Canadian Diabetes Association CPG, 2003). When all other reasons have been checked out, DPN describes nerve loss and injury in people with diabetes (Pop-Busui et al., 2017). The idea encompasses a wide variety of expressions. DPN may manifest as asymptomatic, normal or atypical neuropathic pain, changed or reduced sensations, or conventional neuropathic pain. Early symptoms could be motor or sensory, like atrophy and weakness. DPN that has no signs is not unusual. "Typical neuropathic pain" describes the discomfort felt while wearing gloves and stockings. Peripheral neuropathic pain ranges widely in location and intensity and is covered by atypical neuropathic pain. The individual's standard of life may be negatively impacted by pain that is resistant to treatment.

Finally, altered or reduced sensations may be a symptom of sensory issues. Both of which increase your risk of developing diabetes-related issues such as diabetic foot, ulcers, even amputations. In the diabetic population, DPN problems are significant causes of morbidity and mortality (Gregg et al., 2004). Vibration sensation and diminished proprioception, both of which point to large-fiber function, are at-the-bedside neuropathy symptoms. Additionally, a decrease in pain, sensitivity to light touch, and temperature, which reflect the activities of small fibers, could be an early sign of neuropathy. Slower reflexes and wasting are examples of motor signs. All of the signs and symptoms of motor along with sensory neuropathy are identified as diabetes consequences (Hamed & Monem, 2018). The emphasis of treatment should be on prevention rather than reversibility. Once DPN has manifested, there is presently no treatment that can stop it or alter its course. In T1DM, strict control of glucose levels may be a helpful intervention to prevent or delay the onset of the illness. In both types of diabetes, it may reduce the signs and symptoms of clinical neuropathy (DCCT Research Group, 1995). Through thorough screening, pain management, & foot care, the current aim of DPN treatment is to advance life quality and decrease complications. Oxidative stress and DPN.

Significant metabolic irregularities and pathological changes are brought on by diabetes in many different organs. Oxidative stress has a significant impact on the etiology of diabetes, including diabetic complications (Thorpe & Baynes, 1996). Due to prolonged and chronic hyperglycemia, which impairs the antioxidative defense system's effectiveness and encourages the generation of free radicals, diabetes and experimental animal models face severe oxidative stress (Baynes, 1991). Examples of such models include diabetic rats and mice created by alloxan or STZ (Gillery et al., 1989).

The antibiotic Streptozotocin (STZ), produced by Streptomyces achromogenes, is the one used in experimental diabetes the most frequently (Rakieten, 1963). Currently, it is uncertain how STZ destroys pancreatic -cells and results in hyperglycemia. Similar to the actions attributed to alloxan's diabetogenic activity, STZ has been related to a number of outcomes, including damage to pancreatic cell membranes (Bhattacharya, 1954) and islet cell loss of cytoplasmic nicotinamide adenine dinucleotide (Schein et al., 1973).

Additionally, STZ has been shown to methylate and break DNA strands in pancreatic islet cells (Matkovics et al., 1997).

Both natural and experimental diabetes are known to cause neuropathy, and the STZ model is frequently used to research clinical DPN (Jakobsen & Lundbaek, 1976). Animal studies may give light on the alterations that take place in the early stages of human diabetes, despite the difficulty to recreate the extensive structural impairments in peripheral nerves that have been observed in instances with developed neuropathy in people (Sharma et al., 1985).

Various interactive pathways contributing to the pathogenesis of DPN have been identified in both human and murine models. It is now recognized that prolonged hyperglycemia plays a significant role as a primary risk factor for the development of neuropathy (DCCT Research Group, 1995). Prolonged hyperglycemia results in heightened oxidative stress, elevated aldose reductase (AR) activity, accumulation of advanced glycation end-products (AGEs), and reduced Na+/K+-ATPase activities (Cameron et al., 1997)(Karachalias et al., 2003) among other effects (Gries, 1995). As a result, the peripheral sensory and parasympathetic nervous systems may be gradually impacted (Vague et al., 2004). Given the limitations of current ineffective pharmaceutical treatments, there are limited strategies available to influence or halt the natural progression of DPN aside from strict glycemic control (Thomas's & Diabetic Study Group, 1986). Despite the challenges ahead, there is optimism for more successful diabetes mellitus and related treatments in the near future.

1.2.2. DPN Biomarkers

According to animal models, nerve demyelination and defective Schwann cells are the main causes of DPN. Peripheral myelin protein 22, or Proteolipid protein 1, Myelin basic protein, or myelin protein zero (MPZ) are a few examples of proteins whose expression can fluctuate as the myelination process occurs (Khan, 1999) (Salem, 2005). Human biomarker investigations may offer important information concerning the physiological processes of nerve damage in diabetes in addition to molecular understanding (Salih et al., 2009) (Sayeed et al., 1999). A unique biomarker-based approach adopted from

oncology uses circulating nucleic acids to identify and monitor patients who are at risk, and its diagnostic & prognostic capabilities have been confirmed in numerous trials (Reddy et al., 2018).

Circulating nucleic acids have not been extensively studied in diabetes research to discriminate the extent of the disease (Morrow, 2004) (Kolb et al., 1987). Enhanced diagnostic specificity can be achieved by examining the nucleic acids exclusively expressed by peripheral cells within the nervous system. This concept is grounded in the notion that, when cellular damage or other mechanisms like exosomal secretion occur, neuronal cells safeguard genetic transcripts within the cell and release them into the bloodstream. Another compelling biomarker within the realm of neurology is neurofilament light chain (NfL). It is a constituent present in neurons and axons, detectable in cerebrospinal fluid and, to a lesser extent, in serum/plasma (Wu & Huan, 2007).

NfL offers a unique advantage in evaluating the extent of axonal damage, making it valuable for assessing the severity of inherited neuropathies like Charcot-Marie-Tooth syndrome, ALS, AD, and HD. In this prospective observational pilot study, we explore the potential of serum levels of a newly developed neural damage marker, NfL protein, along with circulating myelin-specific mRNA (cmRNA), as potentially non-invasive tools for identifying markers of DPN and assessing their predictive capability regarding disease progression.

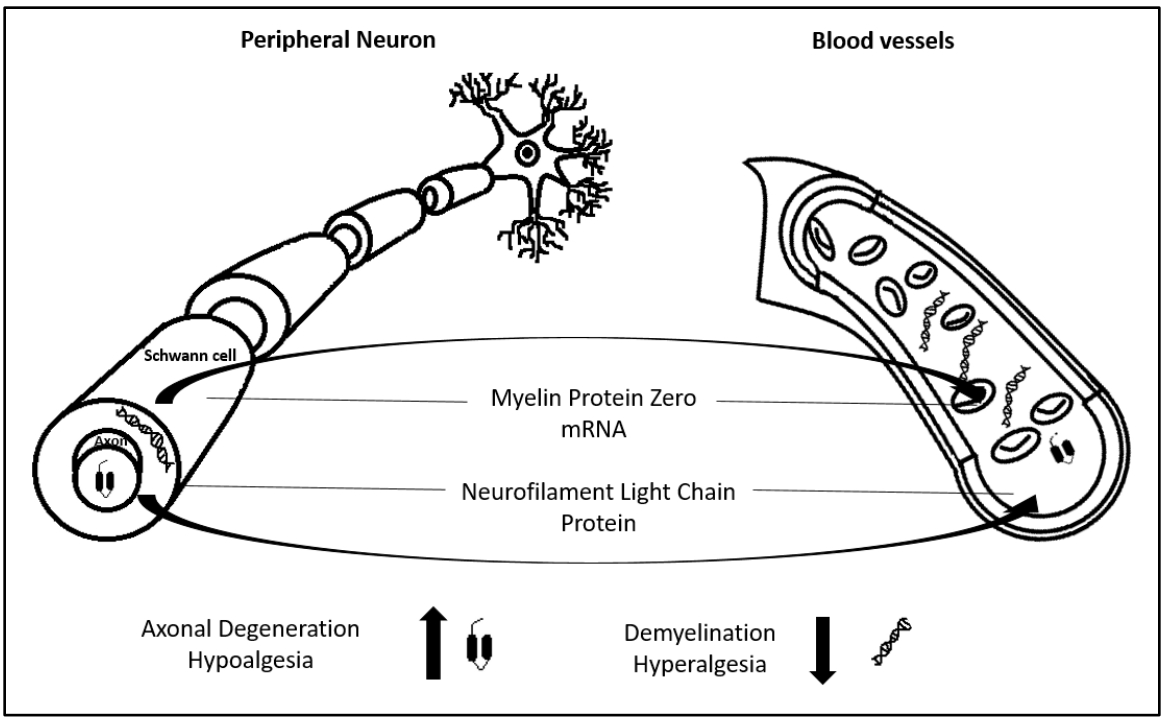

Figure 2: DPN Pathology. NfL and MPZ mRNA are the biomarkers that are not invasive that could be used to help diagnose DPN and its progression.

NfL and MPZ mRNA have been identified as non-invasive biomarkers that may be utilized to assist diagnose DPN and maybe predict its progression through a pilot study in people. We established a link between axonal injury, as determined by higher NfL proteins in the blood, and a hyperalgesic phenotype, However, it is important to note that further extensive multicenter studies involving larger patient populations are necessary to validate our findings. The decreased levels of MPZ cmRNA may indicate demyelination, particularly in subacute cases, which could aid in identifying a hypoalgesic phenotype. Our findings align with a previously proposed theory (Wu & Huan, 2007), suggesting that the molecular underpinnings of pain are distinct from the processes linked to sensory function loss. Consequently, the assessment of MPZ and NfL holds significant promise for investigating the structural changes occurring in Diabetic Peripheral Neuropathy (DPN) and potentially identifying novel treatment targets.

1.3. Medicinal Herbs for treatment and prevention of DPN

Due to safety and increased compliance among patients in treating the various lesions of DPN, natural products are viewed as a potential alternative treatment (Kolb et al., 1987).

Recent studies have revealed that the neuroprotective, antioxidative, and anti-neuroinflammatory characteristics of regularly Plant-derived compounds have shown promise in potentially enhancing the management of DPN (Wu & Huan, 2007). Comprehensive research efforts are underway to explore the therapeutic potential of various plant-derived compounds such as flavonoids, alkaloids, phenolic compounds, terpenoids, saponins, and phytosterol-like components found in herbal remedies from diverse cultures worldwide. These compounds are being investigated as potential novel therapeutic candidates for addressing various diabetes-related issues (Osman et al., 2017).

The natural medicine sector with the most ongoing study into DPN therapy options is EAHM (WHO, 2000). EAHM, which stands for East Asian Herbal Medicine, serves as a collective term encompassing natural substances utilized in research and as medicinal treatments in several East Asian countries, including Korea, Taiwan, China, and Japan (Cho et al., 2014). EAHM distinguishes itself from herbal therapy in other regions of the world by its practice of using similar herbal materials across multiple countries. EAHM operates based on a distinctive prescribing concept that aims to maximize the synergistic effects of polyherbal formulations. Strong clinical and preclinical research supports the efficacy of EAHM, which is still being used as a therapy in the aforementioned countries.Given that EAHM is helpful in treating DPN, it is important to investigate the EAHM components that support DPN mitigation (Jo et al., 2023).

The interplay of metabolic & vascular factors, of which hyperglycemia is the most significant, complicates the fundamental cause of DN. Effective treatments for DN, a frequent, incapacitating condition brought on by nerve damage, include glycemic management and pain relief. Ancient texts like the Charak Samhita and modern pharmacology have both attested to the enormous medicinal value of herbal plants for achieving more comprehensive therapy for a variety of ailments. The application of traditional Chinese medicines (TCM) to cure, diagnose, or regulate human functions is guided by TCM theory. TCM is based on botanicals, which include at least 11,146 species of plants from 383 families and 2309 taxa, together known as Chinese herbal medicines (CHM). CHM contains a number of active ingredients, such as extracts and

monomers for short, which are effective treatments for diabetes and related side effects (Ye et al., 2002).

The species of mulberry (*Morus Alba L.*), is a member of the family Moraceae. Other names for mulberry are Ramulus Mori or Sangzhi (Cardinale et al., 2006). This genus contains 24 species and 100 distinct subspecies. These are primarily found in climatically moderate places, while some have expanded to tropical regions of Africa (Srivastava et al., 2006). Particularly Greece and Turkey, to provide fruit which is utilized in a variety of regional recipes. Several studies have revealed that Morus alba possesses anti-diabetic characteristics.

From ancient times to the present, practically all societies have employed plants as medicine. Based on their novel pharmacological activity modalities, natural substances derived from higher-level plants that have been conventionally utilized as medicines may be a good place to start when creating novel pharmaceuticals. Because they are utilized to make drugs that use less expensive sources and have minimal side effects, medicinal plants are now essential to the global economy. since over 85% of conventional medical procedures employ plants or plant extracts. The secondary metabolites accumulated and produced by medicinal plants include alkaloids, steroids, flavonoids, terpenes, saponins, resins, glycosides, quinines, volatile oils, lactones, and many others. According to a recent estimate by the World Health Organization (WHO), 80% of people worldwide depend on medicinal herbs for a portion of their fundamental health care (Alamgir et al., 2018).

1.3.1. Nigella sativa (NS)

The fennel flower plant, or NS is a natural herbaceous plant of the Ranunculaceae family. Traditional local healers advise regularly using black seeds because they are thought to be one of the best therapeutic mediums available to treat a variety of ailments. The therapeutic potential of Nigella sativa (NS) seed constituents in addressing various human health issues has garnered substantial support through over 150 studies conducted since 1959. NS has a long history of use as a natural remedy for a wide range of conditions, including asthma, chest congestion, hypertension, diabetes, inflammation,

cough, bronchitis, headaches, fever, dizziness, and influenza, as well as for promoting overall health (Shuid et al., 2012).

1.3.2. Cassia angustifolia (CA)

Representatives of the Caesalpiniaceae family include the species of Cassia. The wider family of leguminous plants, Caesalpinideae, is generally thought of as including the subfamily Caesalpiniaceae. Although it belongs to the Papilionaceae and Mimosaceae families, it is distinguished by its five free petals and few stamens. The genus of tropical trees, shrubs, including a few woody plants is known as Caesalpiniaceae. Cassia species hold significant importance in phytochemical and pharmaceutical research due to their notable therapeutic properties and widespread utilization in traditional medicine, particularly for their laxative and purgative effects (Jani & Goswami, 2020).

1.3.3. Phytochemical study of herbs

Both the seeds and leaves of the medicinal plants NS and CA have high concentrations (+++) of alkaloids, carboxylic acids, coumarins, phenols, resins, saponins, and steroids. The DPPH free radical scavenging experiment of NS and CA preparations at three different doses (5, 10, and 20 g/ml) showed that these medicinal herbal plants are great potential antioxidant candidates. The entire study thus proven that these conventional herbal medicines require extensive testing before being included in pharmaceutical formulations (Tanaka & Kashiwada, 2021).

1.4. Induction of T1DM in mice using repeated low doses of STZ

The STZ-induced diabetic mice is a very popular animal model for researching the causes of excruciating diabetic neuropathy and assessing potential novel therapies (Ohnishi et al., 2004). STZ is typically administered in a single high dose and is known for its high specificity as a cytotoxic agent for pancreatic islet cells, often resulting in complete cell necrosis and the onset of diabetes within 48 hours. However, a study by Like and Rossini in 1976 revealed a different pattern when mice were given multiple small doses of STZ over a period of 5 days. In this case, the onset of hyperglycemia was delayed, and it couldn't be attributed to an immediate, direct fatal impact of the drug. Instead, this delay

was associated with kinetic factors. The repeated, low-dose administration of STZ initiated an inflammatory process that further impaired the activity of islet cells, ultimately leading to insulin deficiency and the development of diabetes (Like & Rossini, 1976).

STZ Partially kills pancreatic islets and high blood sugar, this reaction more closely resembles T1DM than a single, high dose STZ treatment in terms of etiology and morphologic changes (Kolb, 1987). Currently, the widely adopted approach for establishing an animal model of Type 1 Diabetes Mellitus (T1DM) involves the repeated administration of low doses of STZ. This method entails giving mice multiple low doses of STZ (at 40 mg/kg, i.p.) over a five-day period. This T1DM model is commonly utilized to assess the efficacy of potential anti-diabetic medications. Screening tests may involve administering the test drug either before or after inducing diabetes, depending on the specific objectives of the research study (Papaccio & Chieffi-Baccari, 1992).

1.5. Aim of Current Study

Aim of the current study is to find out the preventive potential of these two herbs CA and NS for diabetic complications specifically diabetic peripheral neuropathy using different DPN biomarkers. In current study, we are

1. Preparing animal model of T1DM using repeated low doses if STZ induction method.
2. Validating the Animal model through biochemical analysis.
3. Evaluating the preventive role of CA and NS for diabetes related neuropathy in STZ induced diabetic mice.
4. Estimating hyperalgesia through behavioral analysis.
5. Estimating the expression of DPN biomarkers i.e. MPZ and NfL.

Chapter 2
Methodology

2.1. Ethical Approval

All protocols which involved animals as participants were approved by research ethics committee, NUST Institutional review board (IRB) at National University of Sciences and Technology, Islamabad to ensure the protection of the rights, safety, dignity and wellbeing of research participants.

2.2. Preparation of formulation from medicinal herbs

2.2.1. NS extract protocol

NS oil was purchased, which is made from the plant's seed, from a nearby vendor. The oil was given orally once daily at a dose of (400 mg/kg body weight) for a maximum of twelve weeks using intragastric intubation.

2.2.2. CA extract protocol

The leaves of CA were ground in a grinder. The herb powdered (100 g) was dissolved in 400 ml of distilled hot water in 30 minutes. The complex was centrifuged at 3500 rpm for 20 minutes after being filtered with That Whatman No1 filter paper (1442 125, Cytiva, UK). The botanical extracts were cooked down for five hours at 80°C in a hot water bath. Until it was used, the purified extract was kept in a refrigerator at 5°C.

2.3. Preparation of Animal models

Animals from the NUST animal home were removed and acclimatized for a week. Their body weight was assessed one week later. STZ was IP injected into the animals for 5 days in a row. The mice's blood glucose levels were checked nine days after the final STZ treatment. For a period of 12 weeks, the animals received herbal dosages of NS and CA. The body weight of the mice was once more assessed after the fifth week of treatment. Body weight and blood sugar levels were assessed following the end of the dosage period. The 13th week saw the completion of behavioral exams. Animals were dissected after all tests were completed for further PCR and histological testing (He et al., 2011).

In the five days leading up to the start of the experiment, two to five male mice were housed per cage under controlled conditions, maintaining a temperature of 24°C, a humidity level of 55%, and a 12-hour light-dark cycle (with lights on at 8:00 AM and off at 8:00 PM). The mice had unrestricted access to both food and water. It's worth noting that the majority of studies involving STZ-induced diabetic mice predominantly employed male mice. This choice is based on the currently available literature, which suggests that female mice exhibit lower susceptibility to the islet-cell toxin used in the experiment (Kolb, 1987). Six mice were assigned to each group, for a total of 30 people, due to the morbidity associated with STZ therapy. All mice were weighed before being divided into control and experiment groups at random (Furman, 2015).

Figure 3: Mice acclimatization. Mice are divided into groups and free water, food is provided

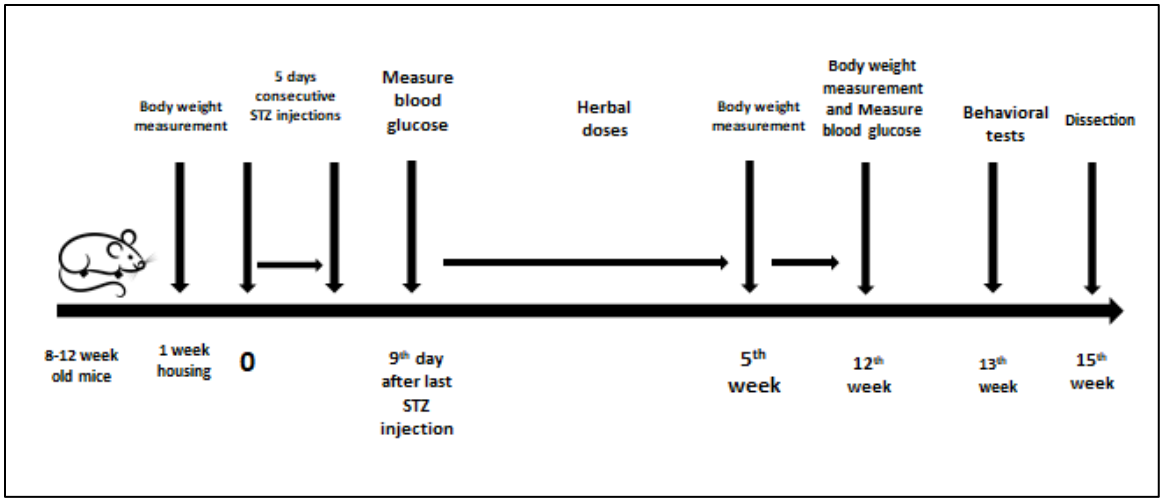

Figure 4: Timeline. Includes induction of DPN, validation of disease, dosages for prevention and dissections.

2.3.1. STZ Treatment to mice for DM induction

All food from all cages was taken out on the first day of the experiment and consumed four hours before STZ therapy. As usual, water is supplied. A single container was utilized for three mice, and within it, 4 mg of STZ was accurately weighed. The STZ was placed in a 1.5 ml microcentrifuge vial, which was then covered with aluminum foil. To create the citrate buffer, a 50 mM sodium citrate buffer with a pH of 4.5 was prepared. Immediately before injection, the STZ was dissolved in this buffer to achieve a final concentration of 4 mg/ml. It is crucial to note that STZ solutions should be freshly prepared just prior to use and administered within 5 minutes of dissolution because STZ undergoes degradation within 15 to 20 minutes after dissolving in the citrate buffer. The administration of the STZ solution was carried out via intraperitoneal injection, using a 1 mL syringe equipped with 25 G needles, following the procedure outlined in Current Protocols by Donovan and Brown in 2006.

40 mg/kg (1 ml/100 g) of the experimental substance was administered. Mice in the control group received intraperitoneal injections of a similar volume of citrate buffer solution (pH 4.5). Intraperitoneally (i.p.) administered dosages of STZ elicit the same reactions (Like & Rossini, 1976). The mice's original housing has been recreated. Access to regular food and drink with 10% sucrose was unrestricted. On days 2 through 5 (the

next four days), actions were taken. On the sixth and final day of the experiment, regular water was substituted with the 10% water with sugar (Arora et al., 2009).

Figure 5: STZ Administration. Intra peritoneal injection of STZ given to mice.

2.3.2. Blood Glucose Test

On trial day 14, which occurred nine days after the final STZ injection, all mice underwent a fasting period lasting for 6 hours (from, for instance, 7:00 AM to 1:00 PM). To verify hyperglycemia in the STZ-treated mice, blood glucose levels were measured using a One Touch Basic blood glucose monitoring device, with blood samples collected from a tail vein. If the blood glucose levels in the STZ-injected mice were found to be above 150 mg/dl (equivalent to 8.3 mmol/L) and/or significantly higher than those of the control mice, it was deemed suitable for research purposes. Frequently, monitoring blood insulin concentrations was deemed unnecessary as blood glucose levels were considered a reliable diagnostic indicator for diabetes. Once diabetes was confirmed, the administration of the test hypoglycemic medication commenced, and control groups that received appropriate vehicle injections, serving as a control, were also included. The duration of treatment with the experimental drug was adhered to as per the experiment's protocol (Togashi et al., 2016).

Figure 6: Hyperglycemic confirmation. Mice blood glucose test from tail blood to confirm hyperglycemia (threshold >150 mg/dl)

2.4. Administration of Doses

For up to 12 weeks, the liquid extract of CA (150 mg/kg body weight) was given intragastric intubation for 84 days, and the NS oil was given orally once daily in a dose of 400 mg/kg body weight.

Figure 7: Oral doses. Herbal doses of CA and NS were administered to mice once daily

2.5. Behavioral tests

Figure 8: Behavioral analysis. Behavioral tests were performed (hot plate test, cold allodynia test)

2.5.1. Hot Plate Test

A combination of cerebral and peripheral pathways is assumed to be responsible for the hot plate hyperalgesic responses. Animals were placed one at a time, using the hot-plate analgesia meter, on a metal surface that was kept at 51°C. Around the hot plate is a transparent plastic fence. The animals were given a week to get used to the hot plate before the test. The animal was removed from the hot plate 60 seconds after it was put there to prevent tissue damage; the amount of time it took for it to respond to the initial signs of paw licking or a leap reaction to avoid the heat was utilized as a measure of pain tolerance. After each measurement, the plate was cleaned with a damp towel to remove any signs of feces and pee (Menéndez et al., 2002).

2.5.2. Tail Flick Test

The tail-flick test was conducted in warm water as follows. The rat's body was wrapped in a soft cloth that was sufficiently stiff to immobilize it. The tail was held in a bath for 1.5 cm while submerged in warm water (53°C), until the tail was removed (caused by a flicking reaction) or signs of struggle appeared. To avoid causing tissue damage, a cut-off time of 10 seconds was established. The test was run four times at thirty seconds each on

each mouse. The overall mean of the last three data used as the basis for calculating tail-flick latency (TFL) (Pinardi et al., 2003).

2.5.3. Cold Allodynia Test

Allodynia caused by the cold was found. Whenever the animals were returned to their natural environment after three testing sessions of five minutes each, separated by ten minutes, the most consistent answers were obtained. The mice were positioned on a metal plate that was cold (4°C) made by Ugo Basile Srl in Comerio, Italy. The total number of times the animal abruptly raised its left hind paw over a 5-minute period was deemed a nociceptive reaction. The number of paw lifts was totaled (Ruan et al., 2018).

2.5.4. Paw Pressure Test

The paw pressure test, also known as the Randall-Selitto test, measures the pressure generated by mechanical stimulation response threshold. In this test, the outer layer of the paw was subjected to increasing mechanical pressure until withdraw or vocalization took place. In this test, the nociceptive threshold were evaluated. Animals were restrained in a plastic cone or cylinder that permitted access to the hind paws throughout this test, which was conducted with a handheld equipment. The method was time-consuming since the animals had to become accustomed to the confining procedure and the testing equipment before the test in order to produce valid findings. Additionally, vocalization served both the endpoint and the threshold (Cunha et al., 2004).

2.6. Dissections

After the mice were given general anesthesia, the skin of the chest, head, and neck was exposed by making a medial incision that extended from the epigastrium towards the limbs. A trapezoid cut was used to release the thorax. A perfusion cannula (23 gauge) was placed into the right ventricle after a tiny incision was made in the top of the left atrium. Passed through normal saline for 5 min, followed by 2-3 min of perfusion with

4% paraformaldehyde (PFA) in PBS prepared with RNase-free water at pH 7.4.

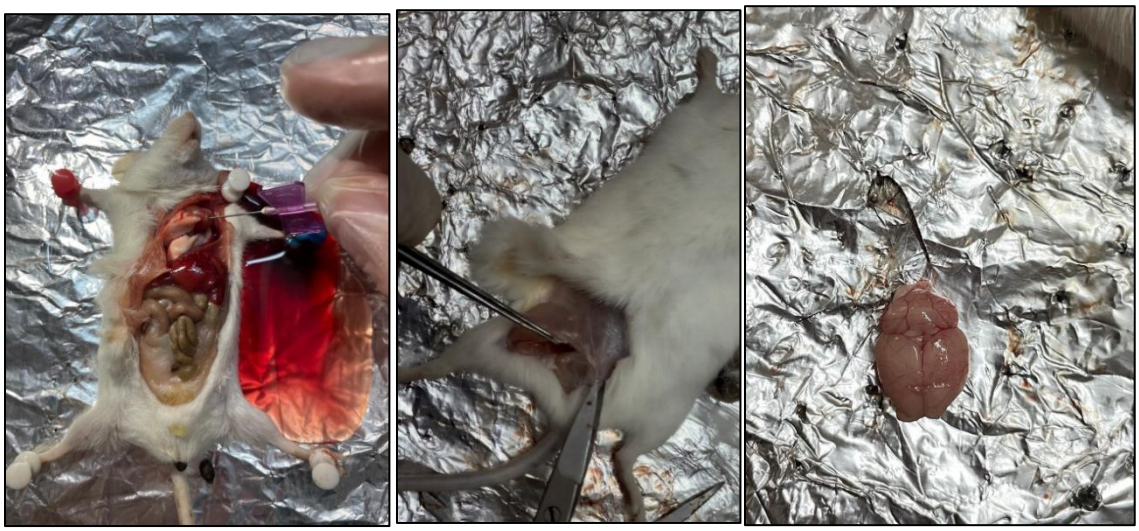

Figure 9: Dissections. A per fusing mice during dissection **B** sciatic nerve extraction **C** extracted whole brain of mice

By gently tugging the tail in the other direction and holding both rear limbs together in one hand, the sciatic nerve was made visible. The nerve's course was followed distally and proximally, and then it was carefully collected. According to the proposed harvested technique and the mid-thigh incision approach, the sciatic nerves were extracted. Due to its length, the removed nerve can be further divided into several sections for independent research such RNA and histochemical/histological study. Sciatic nerve was kept at -80°C right away (Sleigh et al., 2016).

Mice were slaughtered without being perfused in order to extract the RNA. After being removed, the brain and sciatic nerve were both immediately placed in a -80°C freezer. The animal was euthanized through cervical dislocation. Subsequently, the skull was meticulously dissected and bisected along the mid-sagittal plane using fine scissors, commencing from the cerebellum and concluding at the bony region surrounding the olfactory bulbs. Once the skull was opened, the brain was extracted, separated into its left and right hemispheres, and promptly frozen by immersion in finely crushed dry ice. Additionally, as already mentioned, the sciatic nerve was removed. The cryogenic brain and sciatic nerve were transferred to a plastic tube that had already been chilled and kept at -80°C in a refrigerator after a short period of time.

2.7. Histopathological Analysis

2.7.1. Sectioning and Fixation

The specimens of tissue were removed, postfixed for a further 12 hours using the same fixative, and then cryoprotected for a further 24 hours in 30% sucrose. Ten sequential slices (each 25 m thick) were cut from each animal using a cryostat. Some of the pieces were processed for the demonstration. Frozen sciatic nerve paraffination and cutting were incubated for 4 hours in a 90% 2-propanol solution after being exposed to a 75% 2-propanol solution for an overnight period at room temperature (2-propanol, Merck, 109634, diluted with aqua dest.). Then, after being split in half para-sagittally, each hemisphere was kept in 100% 2-propanol (solutions were switched every one hour, two hours, overnight, and one hour again). After that, the substance was put in a rotating stove and heated to 60°C while being mixed with 50% 2-propanol. 50% paraffin (Histowax, Histolab Products AB, Gothenburg, Sweden) (Akbulut et al., 2011).

After 7 hours, 100% paraffin was used in place of the 2-propanol/paraffin mixture. The 100% paraffin had been changed after seven hours, sixteen hours, and seven hours once more. After that, SN was embedded side by side at the Leica Microsystems GmbH's Paraffin Embedded Centre EG1150 in Wetzlar, Germany, with the margins of the para-sagittal cut's cuts facing the subsequent sectional region. The paraffin blocks were next manually cut to a thickness of 5 m using a rotary a microtome (RM2165, Leica Microsystems GmbH, Wetzlar, Germany, and Feather microtome blades type A35), and three sections of each slide were attached to the microtome-cut paraffin blocks. In typical slide boxes, the slides were stored at ambient temperature.

2.7.2. Microscopy

Prepared slides were visualized in Labomed microscope (Labo America Inc. USA) at 10x resolution and snapshots were captured using the software pixel pro. These images were analyzed using software image J version 8.

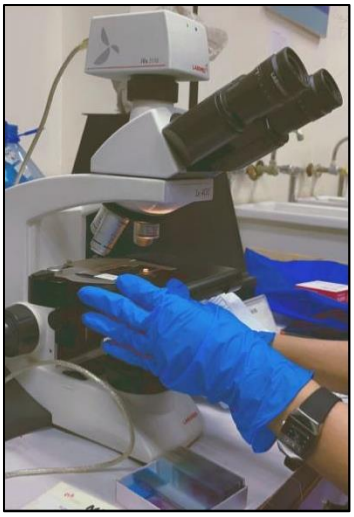

Figure 10: Microscopy. Microscopic images of H&E stained slides of sciatic nerve were taken at 10x resolution

2.8. Molecular Analysis

2.8.1. *In silico* analysis

In order to anticipate the associated binding energies, binding configurations, and binding affinities, molecular docking entails the forecasting of noncovalent interaction between a proteins [Pdb excession numbers **21971831** for MPZ (3OAI) and **22723690** for NfL (NEFL)] and a ligand ligands [thymoquinone, carvacrol, t-anethole, 4-terpineol from NS and Quercimeritrin, rutin, scutellarein from CA]. The ligands were docked with the macromolecules using PyRx's integrated Vina Wizard tool to accomplish this. The results for all of the proteins and ligands listed in the table were then obtained. It is important to note that AutoDock Vina uses multithreading on multi-core processors and improves both the accuracy and the specificity of the binding molecules (Trot & olson, 2010).

2.8.2. Protein-Ligand Interaction

The objective of the current work was to examine the protein-ligand interaction by doing a thorough investigation of the complex using a variety of computational methods. Initially, a PDB file of the complex was retrieved from PyRx and subsequently utilized in BIOVIA Discovery Studio. This was done to generate a three-dimensional visualization as well as a two-dimensional image, illustrating the bonding patterns between the protein

and the ligand. Following this, PyMOL was employed to construct a protein-ligand complex.

2.8.4. Drug Screening

This study applied a strict methodology to the ligand screening process. Particularly, thymoquinone, carvacrol, t-anethole, and 4-terpineol were identified as suitable active compounds of NS and were then obtained from PubChem in the 2D sdf format. Quercimeritrin, rutin, and scutellarein were recognized as suitable active compounds of CA. The drug compounds were then docked with the protein structures MPZ and NFL using Autodocking Vina Wizard in PyRx, followed by 3D visualization using Discovery Studio. Also obtained was a 2D picture showing the relationship between the complexes. PyMOL was used to visualize the drug-protein combination in order to learn more about it.

Additionally, using PyRx's AutoDock Vina Wizard, the medicines were docked to the Protein-Ligand complexes for all of the three proteins. The same procedure was repeated to produce pictures of the Drug-Protein-Ligand complex and their interactions during bonding in both PyMOL and Discovery Studio. Overall, this systematic methodology enabled a thorough assessment of the medication-protein interactions, which may be helpful in upcoming drug development initiatives.

2.8.5. Primers designing

After carefully examining numerous research publications, primers were chosen because of their applicability to diabetic neuropathy. In Blast, the primer sequence were confirmed. Primers were created specifically for the mouse genome and with a low GC content. Using nucleotide blast, the PCR product length was determined and is shown in the table.

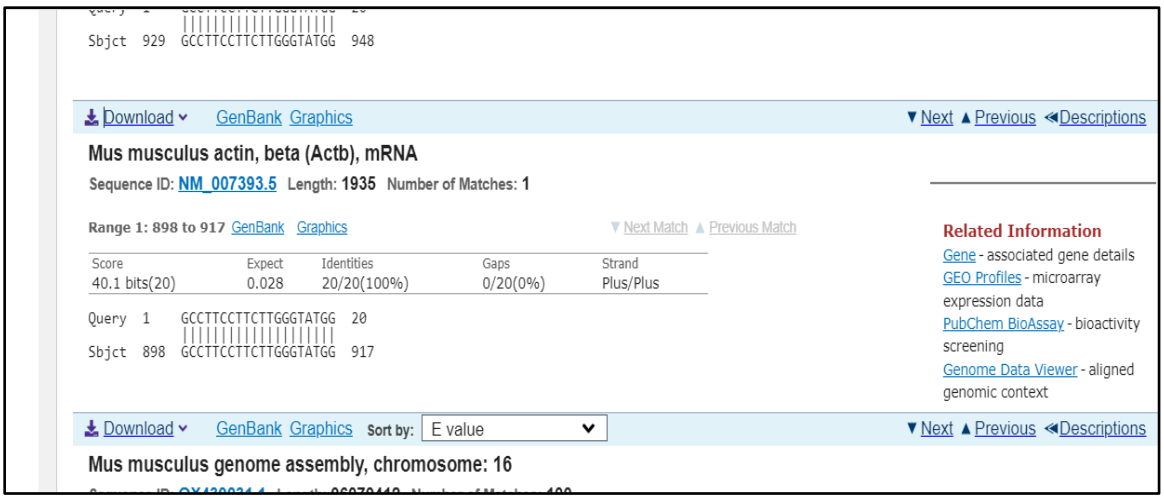

Figure 11: BLAST. Beta-actin forward primer blast with mus musculus genome

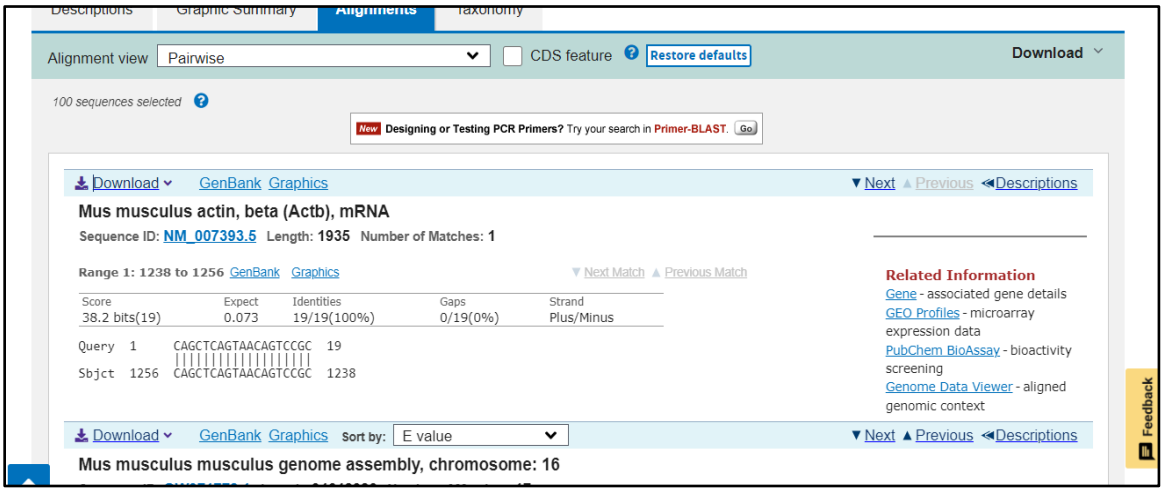

Figure 12 : BLAST. Beta-actin reverse primer blast with mus musculus genome

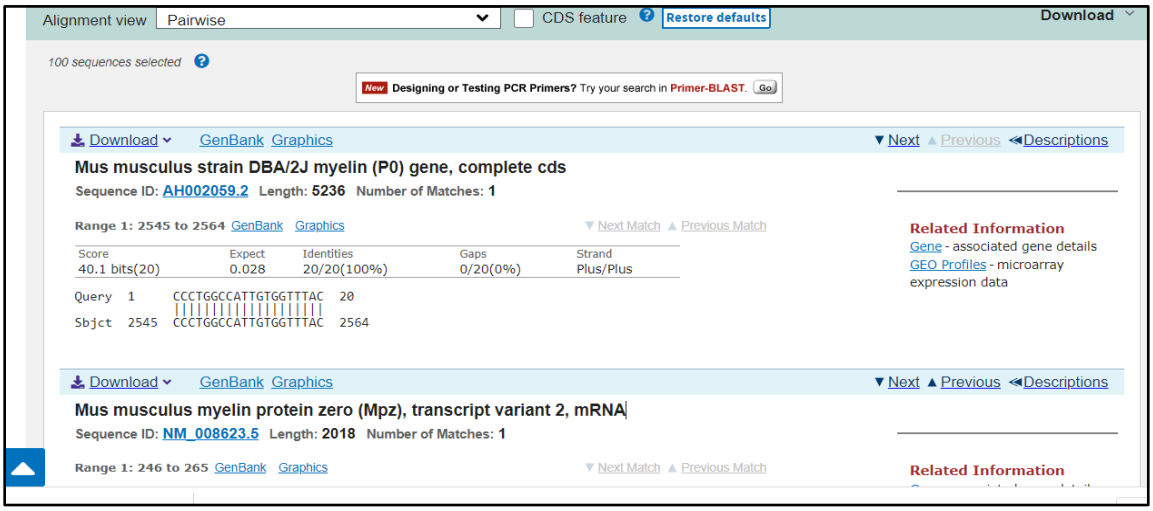

Figure 13: BLAST. MPZ forward primer blast with mus musculus genome

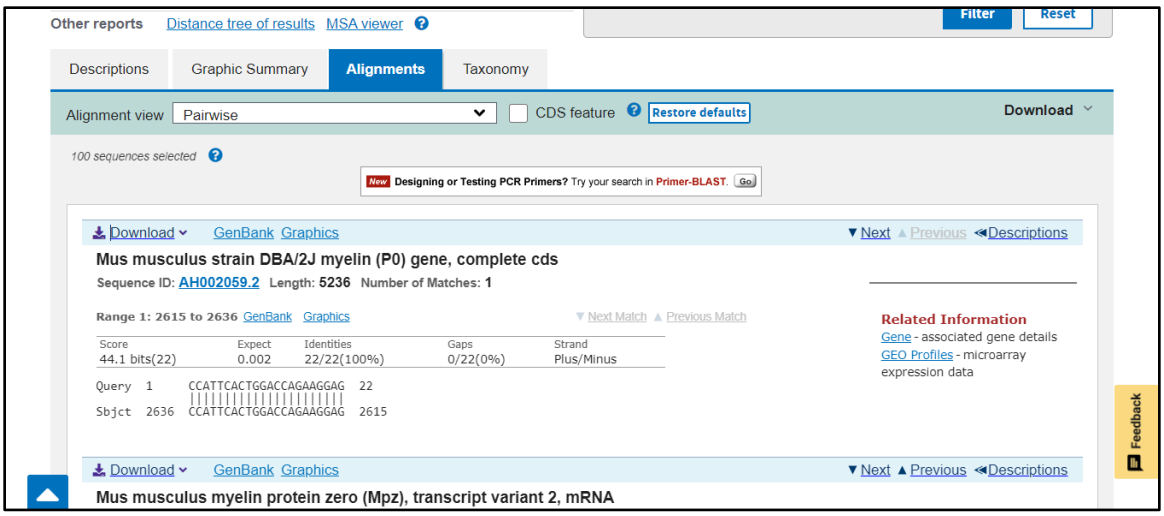

Figure 14: BLAST. MPZ reverse primer blast with mus musculus genome

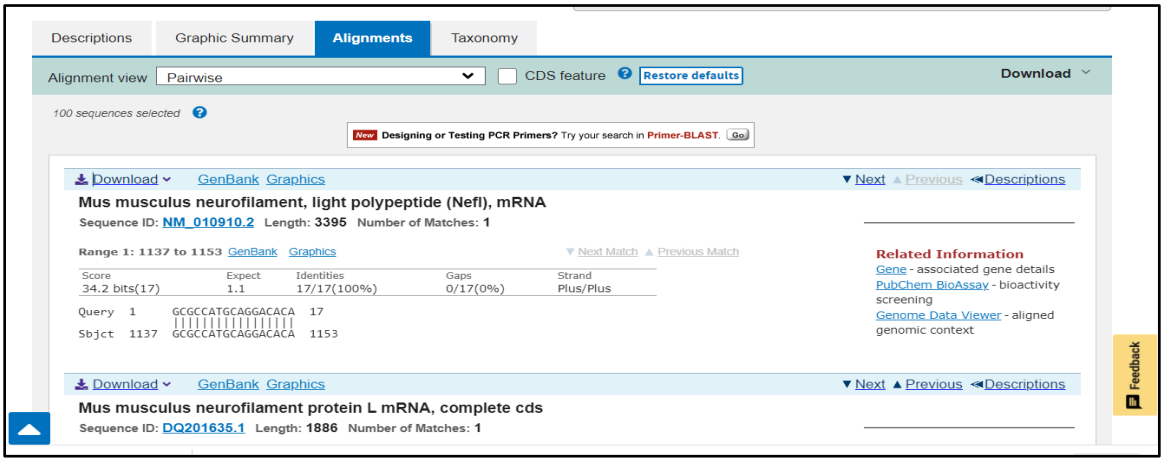

Figure 15: BLAST. NfL forward primer blast with mus musculus genome

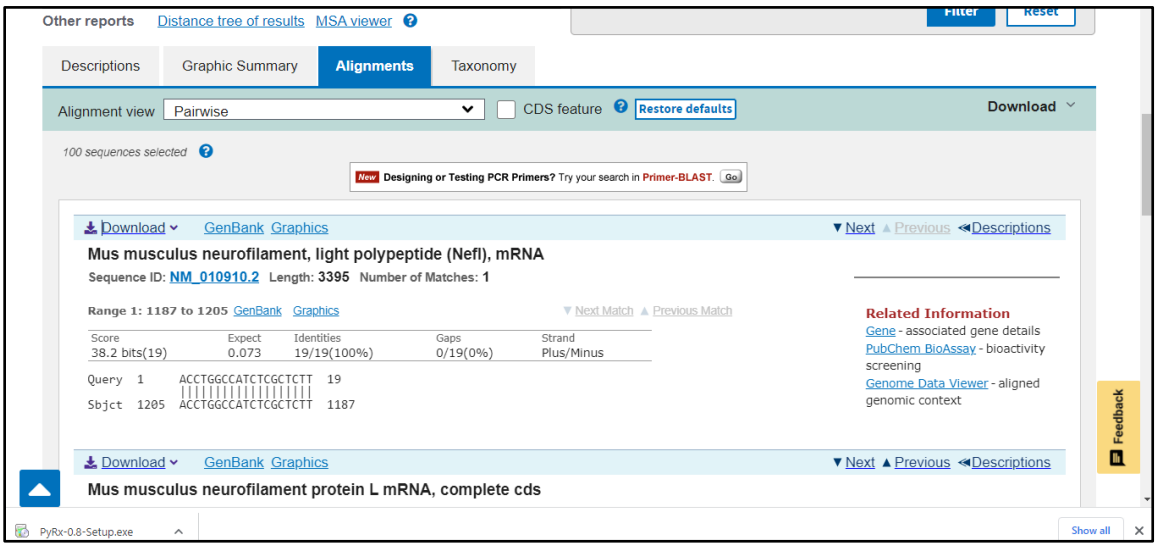

Figure 16: BLAST. NfL reverse primer blast with mus musculus genome

Using the BLAST method, the primer sequence was chosen and verified (Table 1; Figures 11, 12, 13, 14, 15 and 16). The Mus musculus (taxonomy:10090) gene was tested using the primer sequence. In Nucleotide BLAST, the mRNA with the highest degree of specificity and homology was chosen.

Target	Forward	Reverse	Amplicon size [bp]	PCR product length [bp]
Beta-actin (mice)	GCCTTCCTTCTTGGGTATGG	CAGCTCAGTAACAGTCCGC	112	1256-898 =358
MPZ (myelin protein zero) mice	CCCTGGCCATTGTGGTTTAC	CCATTCACTGGACCAGAAGGAG	92	2615-2645 =70
NFL (neurofilament light chain protein) mice	GCGCCATGCAGGACACA	ACCTGGCCATCTCGCTCTT	69	1205-1137 =68

Table 1: Forward and reverse primer sequences. The forward primer sequence is having the product lengths calculated of all the primers

2.8.7. RNA extraction

RNA extraction was carried out using TRIzol Reagent (FineBiotech Cat No.: FTR-100), and the procedure involved specific steps. For each 50-100 mg of tissue sample, 1 mL of TRIzoL Reagent was added. The tissue sample was homogenized thoroughly, either using a power homogenizer or a glass homogenizer. It was crucial to process or freeze the tissue samples promptly upon collection. After allowing the homogenized sample to sit at room temperature for five minutes to ensure the complete dissociation of the nucleoprotein complex, an additional centrifugation step at 12,000 g for 10 minutes at 4°C was necessary for samples with a high concentration of proteins, polysaccharides, or extracellular material to remove insoluble components. The resulting cleaned supernatant was then transferred to a fresh tube for further procedures.

For every 1 mL of the homogenization agent TRIzol Reagent, add 0.2 mL of chloroform. Cap off the tube firmly. For 30 seconds, vigorously shake the tube. At room temperature, incubate for two to three minutes. The sample should be centrifuged at 12,000 g for ten minutes at 4 °C. The aqueous phase is pipetted into a fresh tube. When withdrawing the aqueous phase, try not to draw any of the organic layer or interphase into the pipette. For every 1mL of the TRIzolT Reagent used for homogenization, add 0.5 mL of 100 percent isopropanol to the liquid phase. Ten minutes of incubation at room temperature. 10 minutes of 12,000 g centrifuging at 4°C. Just the RNA pellet should remain in the tube after removing the supernatant. 1 mL of ethanol with a concentration of 75% washes the particle. After quickly vortexing the sample, the tube was centrifuged at 12000 g for several minutes at 4°C. Throw away the washer buffer. For 5 to 11 minutes, vacuum or let the RNA pellet air dry. The RNA pellet should be redissolved in 20–50ml of RNase-free water. Continue with the subsequent application or preserve at -80°C.

Figure 17: RNA extraction. Using Trizol method good quality of RNA was extracted

2.8.8. RNA quality validation

The RNA specimens were spun up at 12000 rpm for 5min prior to the production of complementary DNA. The pellet had been reconstituted in 30 ul of PCR water after the supernatant was discarded. To ensure their integrity, 2% agarose gel was used to run all

RNA samples. WealtecDolphen Doc (S/N470883, Wealtec Bioscience Co., USA) was used to view the gel. The proportion of 28S to 18S rRNA on the agarose gel was checked to make sure it was consistent across all samples.

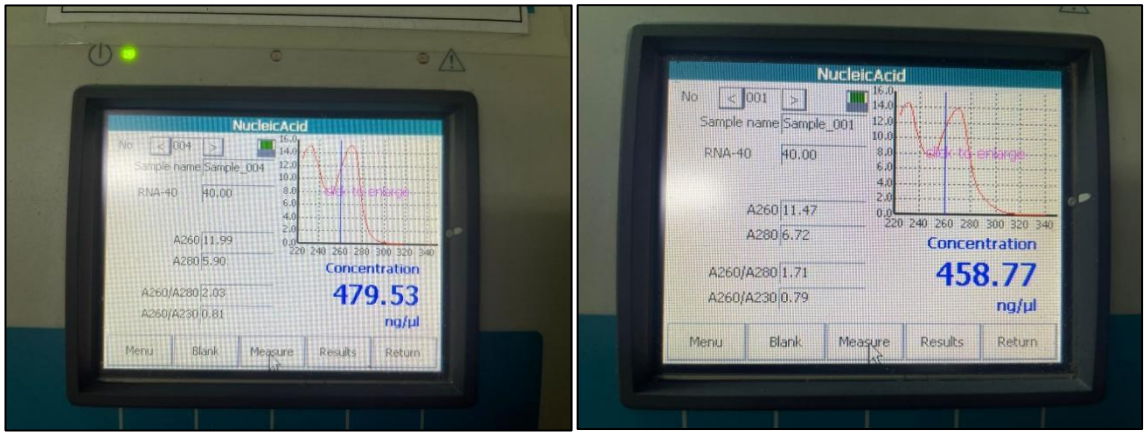

Figure 18: RNA quality confirmation. Nano drop results showing good concentration and quality of extracted RNA

2.8.9. Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for cDNA Synthesis

Utilizing Nanodrop 2000 (Thermoscientific, USA), the extracted RNA was measured, and an identical volume of RNA (2 ug) was converted into cDNA. 4.5 ul of 10 mM dNTPs, 4.5 ul of 5 mM oligodT, and the template were added to a reaction mixture, which was then heated at 55°C for 5 min. After the heat shock, 3 ul of MMLV-RT enzyme, 12 ul of 5x RT buffer, and 6 ul of 0.I M Dithiothreitol (DT'T) were added. By adding PCR water, the total volume of the reaction mixture was increased to 60ul. For the transcription of RNA, the thermocycling temperatures used were 37°C for 10 min, 42°C for 1 hour, and 95°C for 10 min.

2.8.10. Gradient Polymerase Chain Reaction (PCR) for primer optimizations

PCR was carried out using a Labnet PCR thermocycler (Labnet International Inc.). The DNase-free water was used to increase the amount of the reaction mixture, which contained 12.5ul of pcr master mix (wizpure), 1ul forward and reverse primers, and 2ul of cDNA template. The temperature gradient annealing temperatures were 54, 56, 57, 58, 60, 61, and 62°C for 1 minute each, followed by 1 minute at 72°C, with a final dissociation step. The thermocycling protocol used consisted of an initial step at 50°C for

2 minutes, followed by a denaturation step at 95°C for 10 minutes. This was followed by 40 cycles, each consisting of 30 seconds at 95°C. To ensure the reliability of the reaction, each sample underwent the amplification process twice. The purity of the PCR product was assessed using agarose gel electrophoresis.

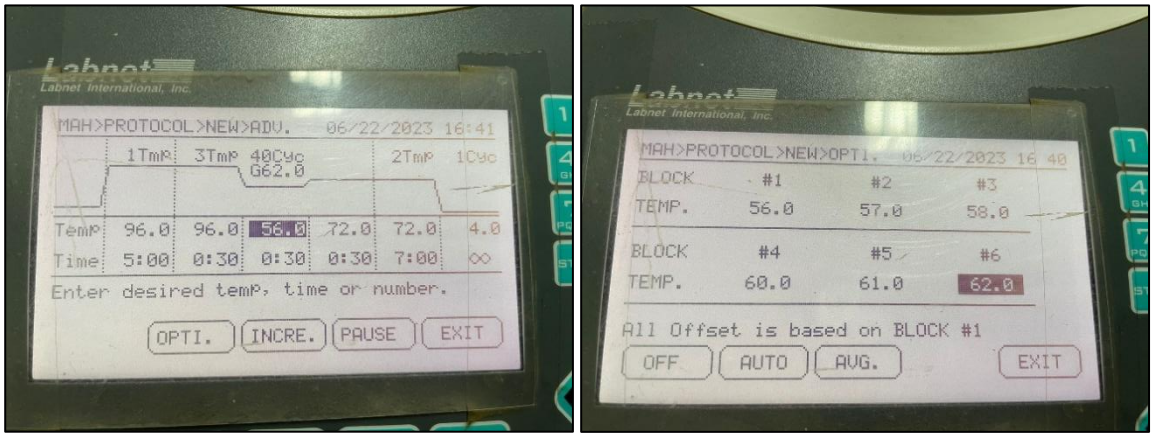

Figure 19: Gradient PCR. Thermocycling conditions provided for gradient PCR optimizations

2.8.11. Gene Expression Analysis by Quantitative Real Time Polymerase Chain Reaction (qPCR)

Real-time PCR was carried out using the AB Quant Gene detection system. After preparing a reaction mixture including 4ul of Syber green qPCR Mix Plus, 1 ul of particular reverse and forward primers, and 1ul of cDNA template, the volume was increased to 20 ul using DNase-free water. The thermocycling settings were 50°C for two minutes, 95°C for ten minutes, 40 cycles of 30 s at 95°C, one minute at (61°C for beta-actin and NfL, 62°C for MPZ), 1 min at 72°C, and then a final dissociation step. For the purpose of ensuring the consistency of the reaction, each sample was run twice. Dissociation curves were utilized to evaluate the PCR product's quality. The values obtained from these trials were examined in relation to the expression of genes data using the Delta CT method after all values were adjusted to those acquired for -actin (Livak & Schmittgen, 2001).

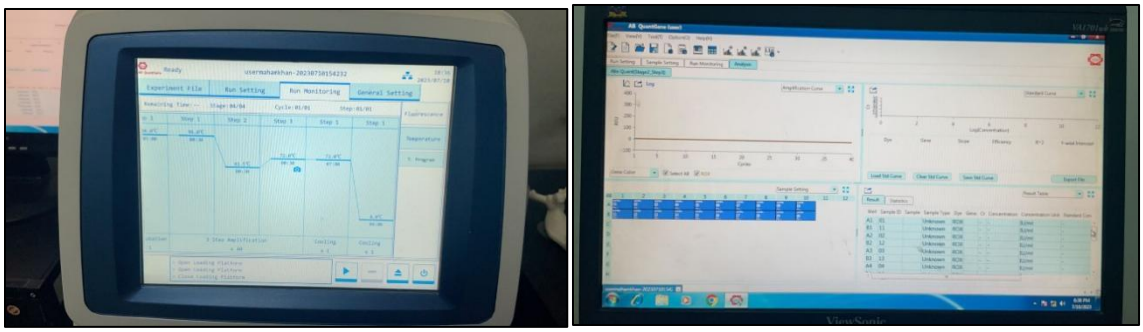

Figure 20: q-PCR Analysis. Conditions provided for q PCR and results output

2.9. Statistical Analysis

Software called Graph Pad Prism version 10 was used to do the statistical analysis. For statistical analysis, one-way ANOVA and Tukey's multiple comparison test were utilized. SD was shown in the error bars (*p <0.05, **p <0.01, ***p <0.001, ****p <0.0001).

Chapter 3
Results

3.1. In Silico Analysis

3.1.1. Molecular docking analysis

From a literature review, the target proteins, proteins MPZ and NfL with the PDB IDs 3OAI and NEFL, respectively, and the ligands, compounds from the two herbs, CA (quercimeritrin, scutellarein, rutin) and NS (thymoquinone, carvacrol, t-anethole, 4-terpineol), were chosen. In PyRx, molecular docking was completed, and the binding energies that were discovered are listed in Table. With the aid of PyMOL and Discovery Studio, 2D visualizations were created. The table lists the sequences with the highest binding energies.

Herbal Extract	**Ligand**	**Target Protein**	**Binding Energy**
Cassia Angustifolia	quercimeritrin	MPZ	-8.7
	scutellarein	MPZ	-7.7
	rutin	MPZ	-9.6
Nigella Sativa	thymoquinone	MPZ	-5.5
	carvacrol	MPZ	-5.6
	t-anethole	MPZ	-5.4
	4-terpineol	MPZ	-4.8
Cassia Angustifolia	quercimeritrin	NfL	-5.5
	scutellarein	NfL	-5.3
	rutin	NfL	-5.2
Nigella Sativa	thymoquinone	NfL	-3.9
	carvacrol	NfL	-4.2
	t-anethole	NfL	-3.9
	4-terpineol	NfL	-3.4

Table 2: Ligands with MPZ & NfL and their binding energies. Compounds of *Cassia angustifolia* and *Nigella sativa* were docked with MPZ & NfL, and binding energies are listed with highest binding energies highlighted in bold.

3.1.2. Binding energy graphs

According to PyRx Vina, quercimeritrin and rutin, two components of CA, have the highest binding energies with the target proteins MPZ and NfL, respectively. Carvacrol exhibits the maximum binding energy with both MPZ and NfL in the case of NGS.

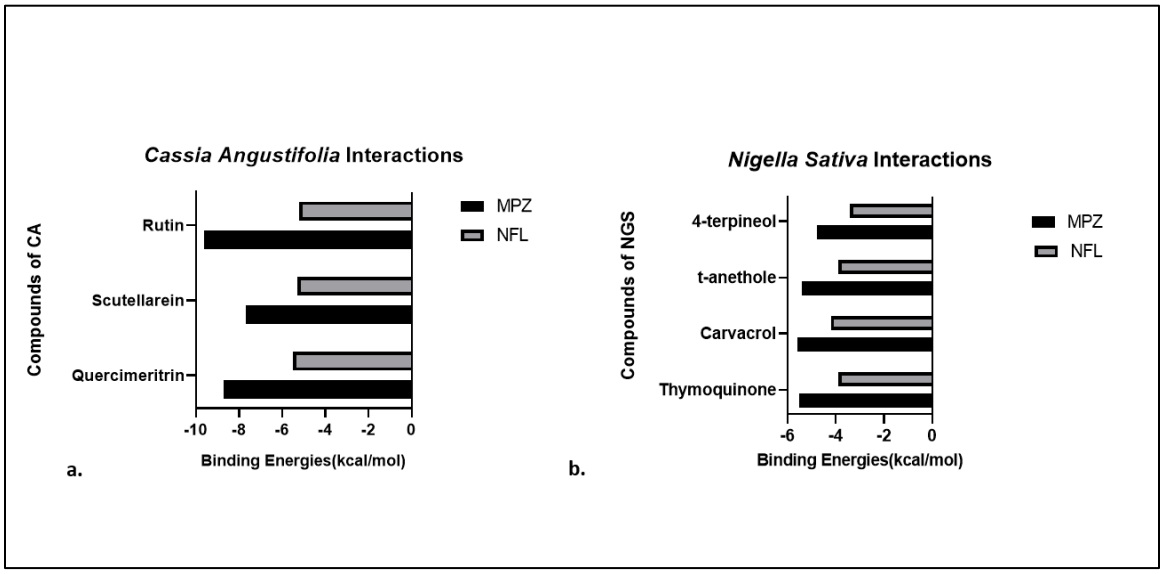

Figure 21: Binding Energy graph of MPZ and NfL with compounds of CA and NGS. A Binding energy graph of MPZ and NfL with compounds of CA **B** Binding energy graph of MPZ and NfL with compounds of NGS. Binding energies evaluated by PyRx are shown in the graph. Rutin is shown having the highest binding energy at -9.6 kcal/mol and 4-terpinole shown having the lowest binding energy of -3.4 kcal/mol.

3.1.3. Molecular Interaction Analysis

PyMOL was used to create a 3D visualization of the protein-ligand complex, and Discovery Studio was used to create intricate 2D diagrams illustrating the interactions and bonds between the protein's structure and the ligand.

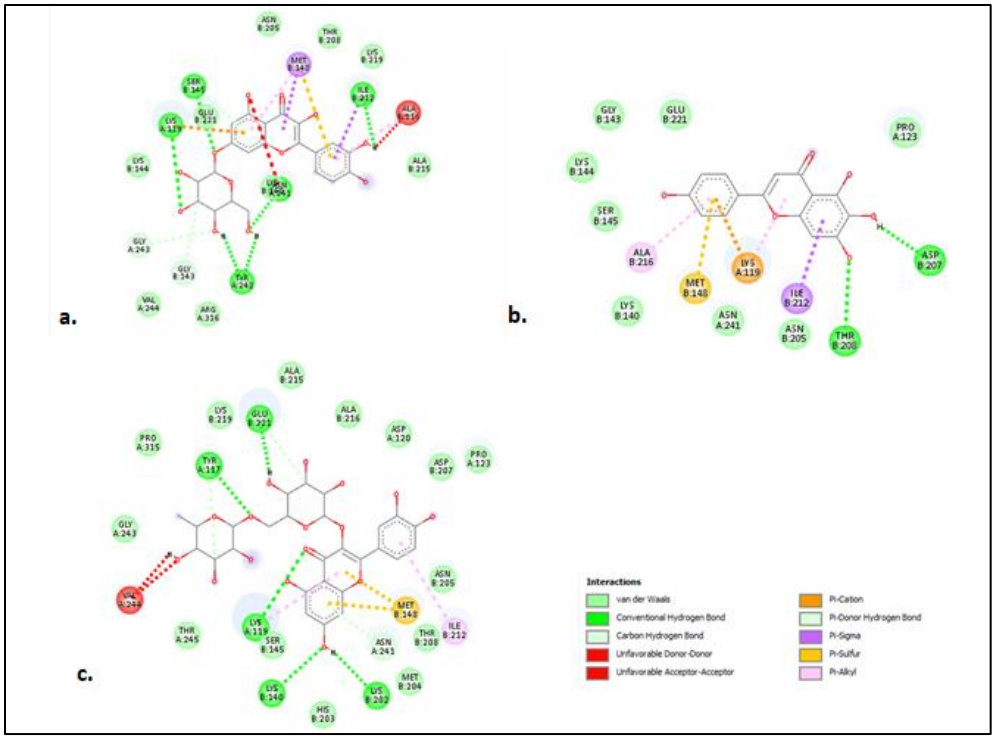

Figure 22: 2D Analysis of MPZ with compounds of *Cassia angustifolia.* A Quercimeritin showing interaction with protein MPZ **B** Scutellarein showing interaction with protein MPZ **C** Rutin showing interaction with protein MPZ.

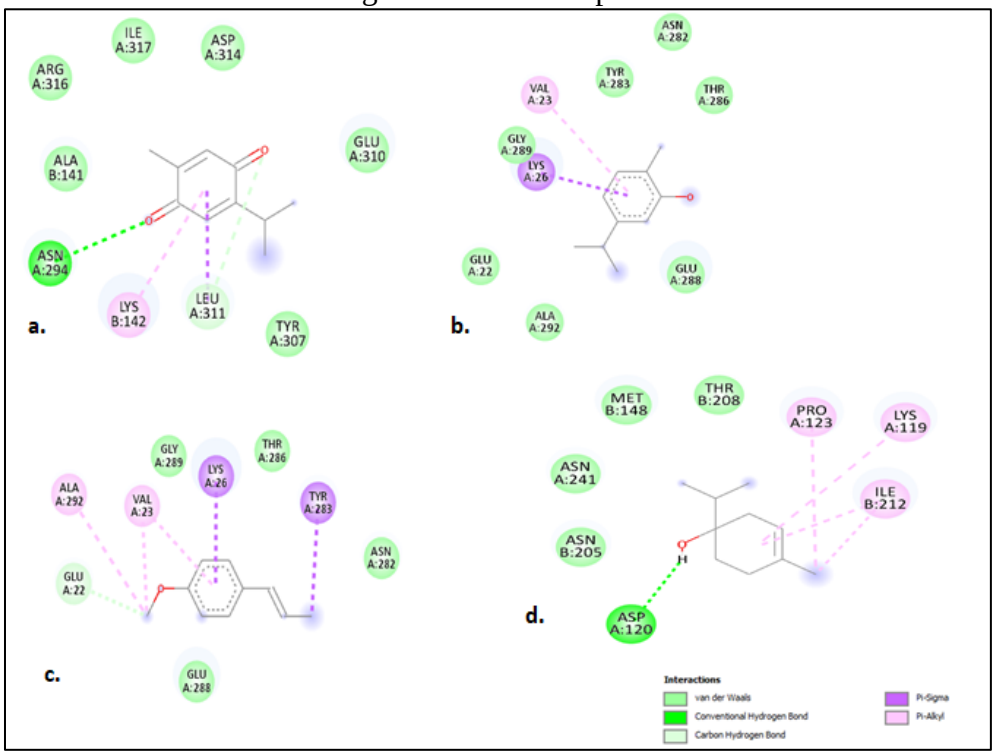

Figure 23: 2D Analysis of MPZ with compounds of *Nigella sativa.* A Thymoquinone showing interaction with MPZ **B** Carvacrol showing interaction with MPZ. **C** t-anethole showing interaction with MPZ. **D** 4-terpinole showing interaction with MPZ.

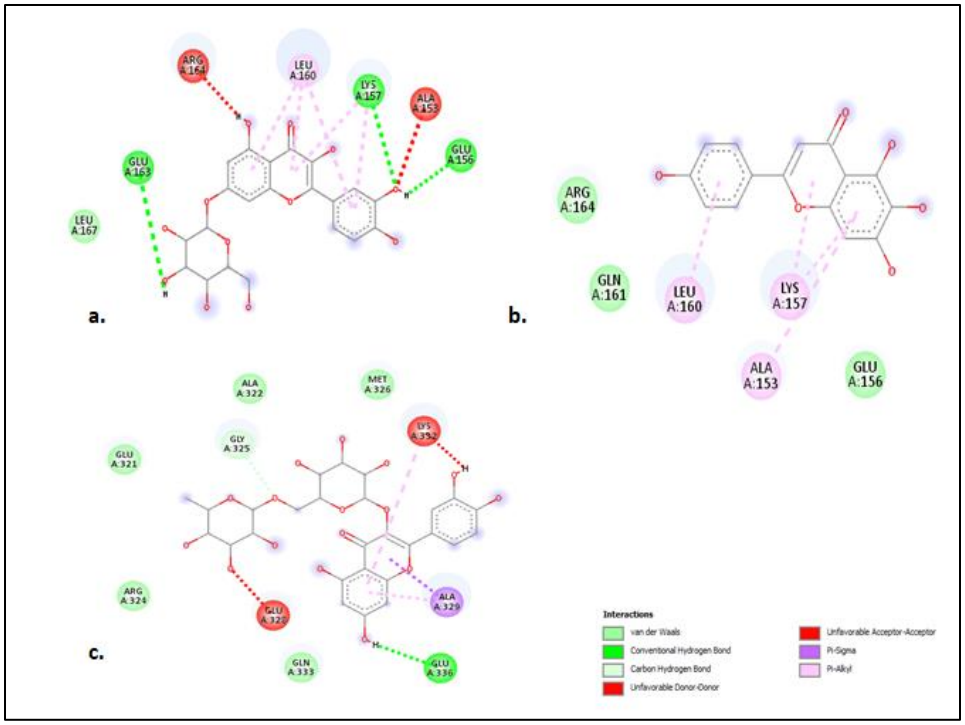

Figure 24: 2D Analysis of NfL with compounds of *Cassia angustifolia.* A Quercimeritin showing interaction with protein NfL **B** Scutellarein showing interaction with protein NfL **C** Rutin showing interaction with protein NfL.

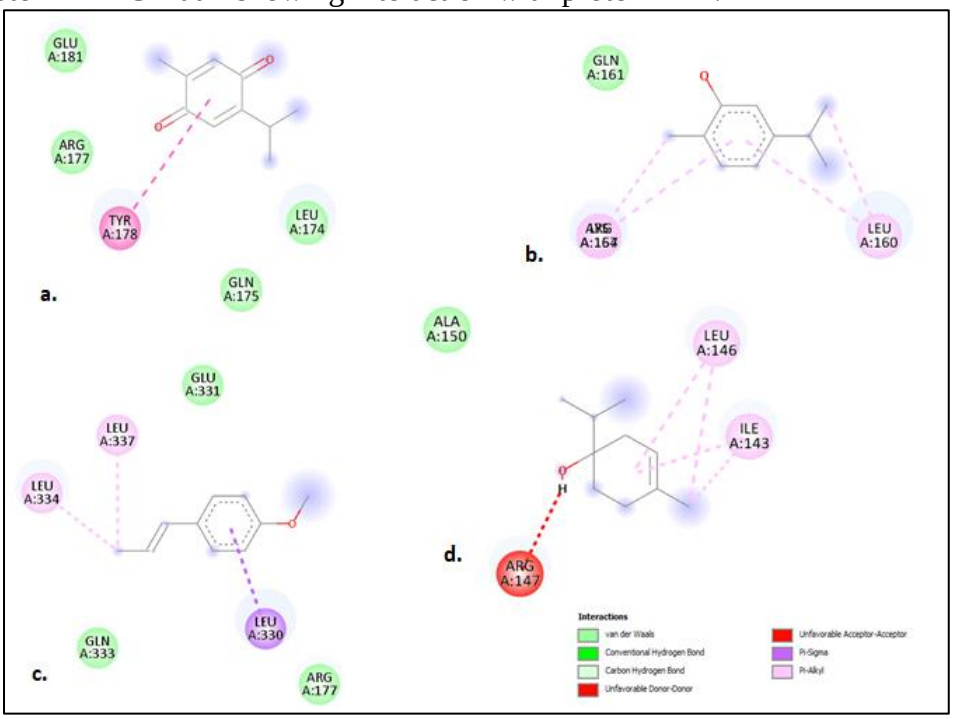

Figure 25: 2D Analysis of NfL with compounds of *Nigella sativa.* A Thymoquinone showing interaction with NfL **B** Carvacrol showing interaction with NfL. **C** t-anethole showing interaction with NfL. **D** 4-terpinole showing interaction with NfL.

3.2. Biochemical Analysis

The arterial blood sugar levels of test animals are shown. Insulin insufficiency and hyperglycemia in diabetic mice were constant P <0.01 and P <0.001, respectively. In the STZ-produced diabetic mice, NS and CA therapy both significantly reduced elevated serum glucose (P <0.01, P <0.05, respectively). At the beginning of the trial, the mice's initial weight were comparable across all groups. Animals with diabetes lost weight after finishing the therapy. Control mice, NS, CA, and combination diabetic animals did not differ in their baseline and final body weights. (Fig 26).

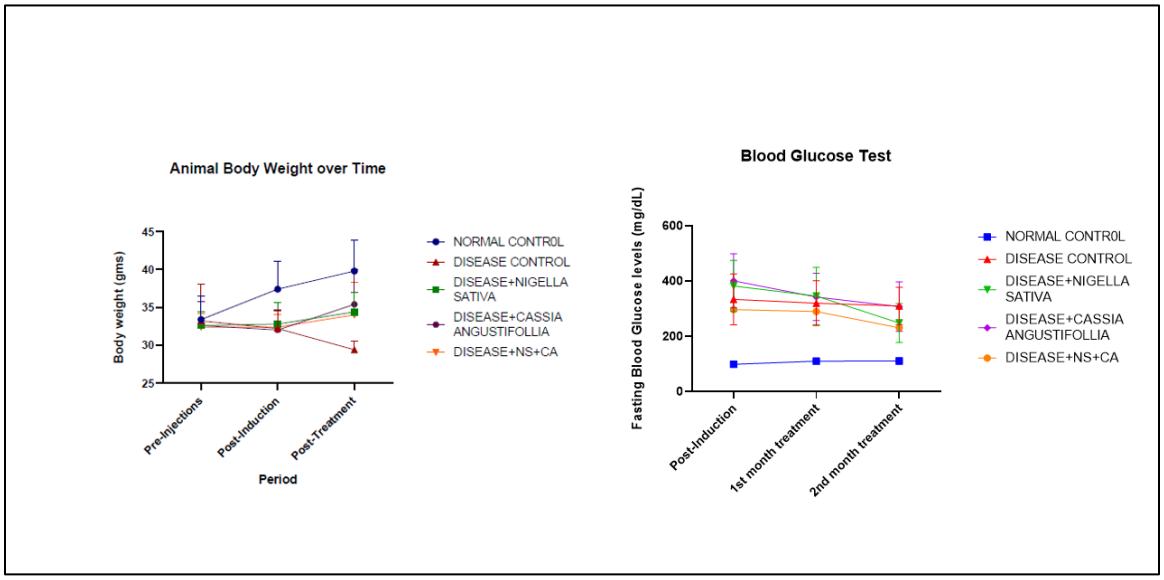

Figure 26: Animal body weight and blood glucose levels, pre and post induction of disease. the diabetic mice showed insulin deficiency and hyperglycemia throughout the study. The initial weight of the mice was similar across all the groups during the trial. The diabetic animals lost weight after the treatment, whereas the control mice, NS, CA, and combination animals did not demonstrate any difference in their baseline and final body weights.

3.3. Behavioral Tests

3.3.1. Hot plate Hyperalgesia

The nociceptive threshold was significantly influenced by NS, CA, and CA+NS in normal mice (n=6 for each dose, P <0.05). Significant reductions in threshold for pain latency were observed in diabetic mice when compared to normal, healthy mice; these reductions were dramatically and dose-dependently reversed by NS, CA, and CA+NS.

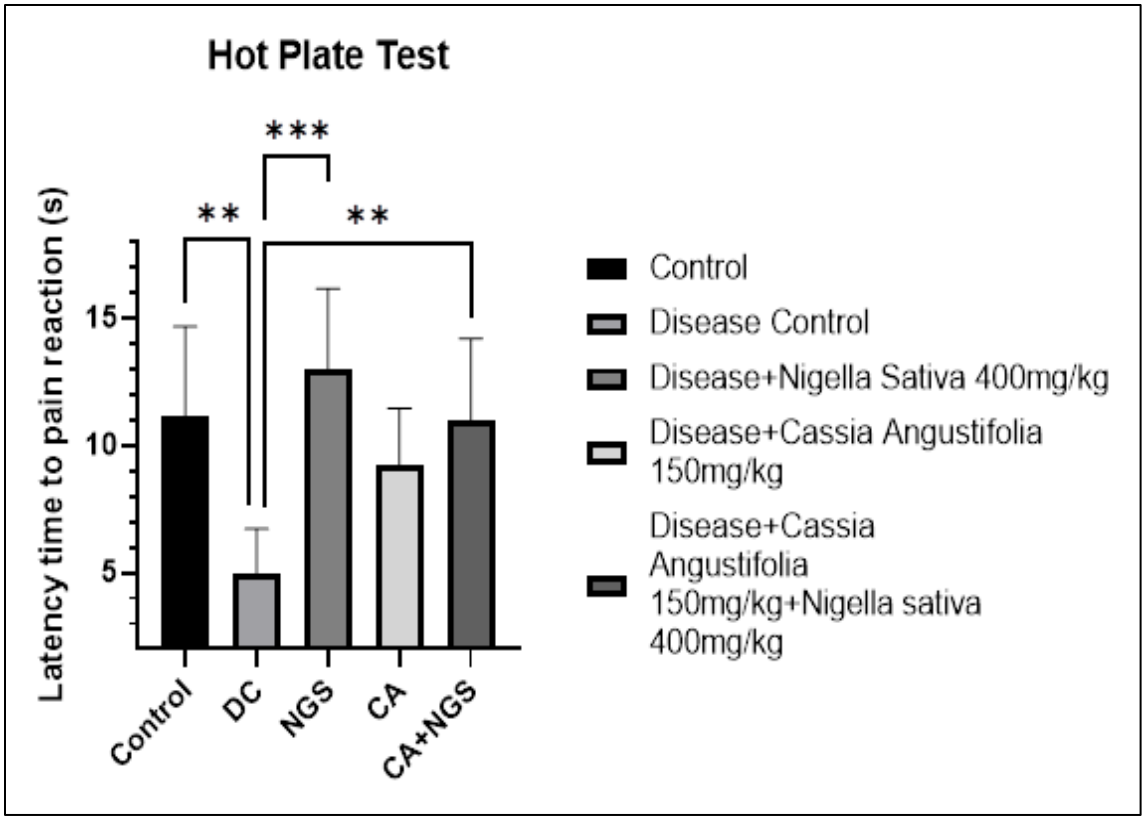

Figure 27: Latency time to pain reaction recorded. When compared with the disease controls, the outcomes of the hot plate hyperalgesia examination demonstrated augmented hot-plate latencies.

3.3.2. Cold Allodynia Test

When mice were administered NS or CA+NS, the total number of paw lifts was considerably lower than when mice were given STZ as a control. When compared to STZ control mice, paw lifts in CA-treated mice increased somewhat but not significantly following treatment. When contrasting with the control group of diabetics, the cold allodynia assessment disclosed a diminished quantity of Paw elevations.

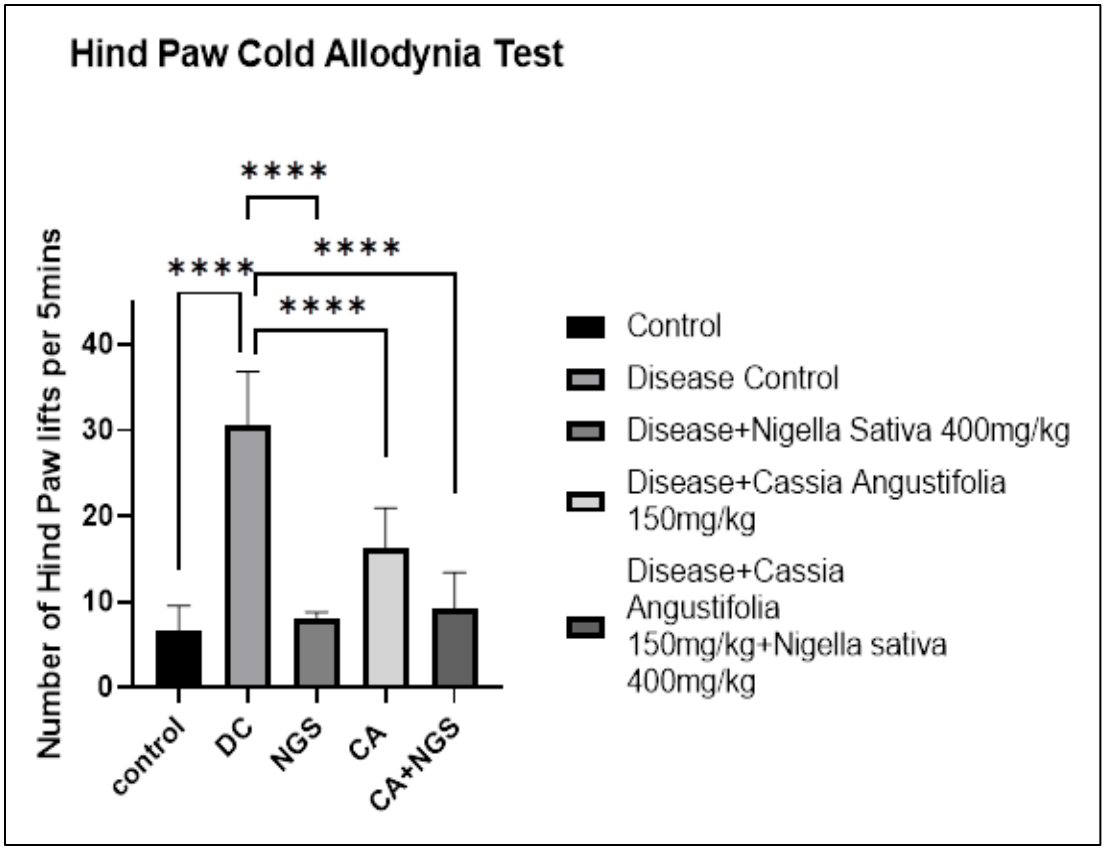

Figure 28: Number of Hind Paw Lifts recorded per 5mins. When contrasting with the control group of diabetics, the cold allodynia assessment disclosed a diminished quantity of Paw elevations.

3.3.3. Paw Pressure Test

Pain threshold latency was reduced in diabetic mice when compared to normal, healthy animals. These reductions were significantly and dose-dependently reversed by NS, CA, and CA+NS.

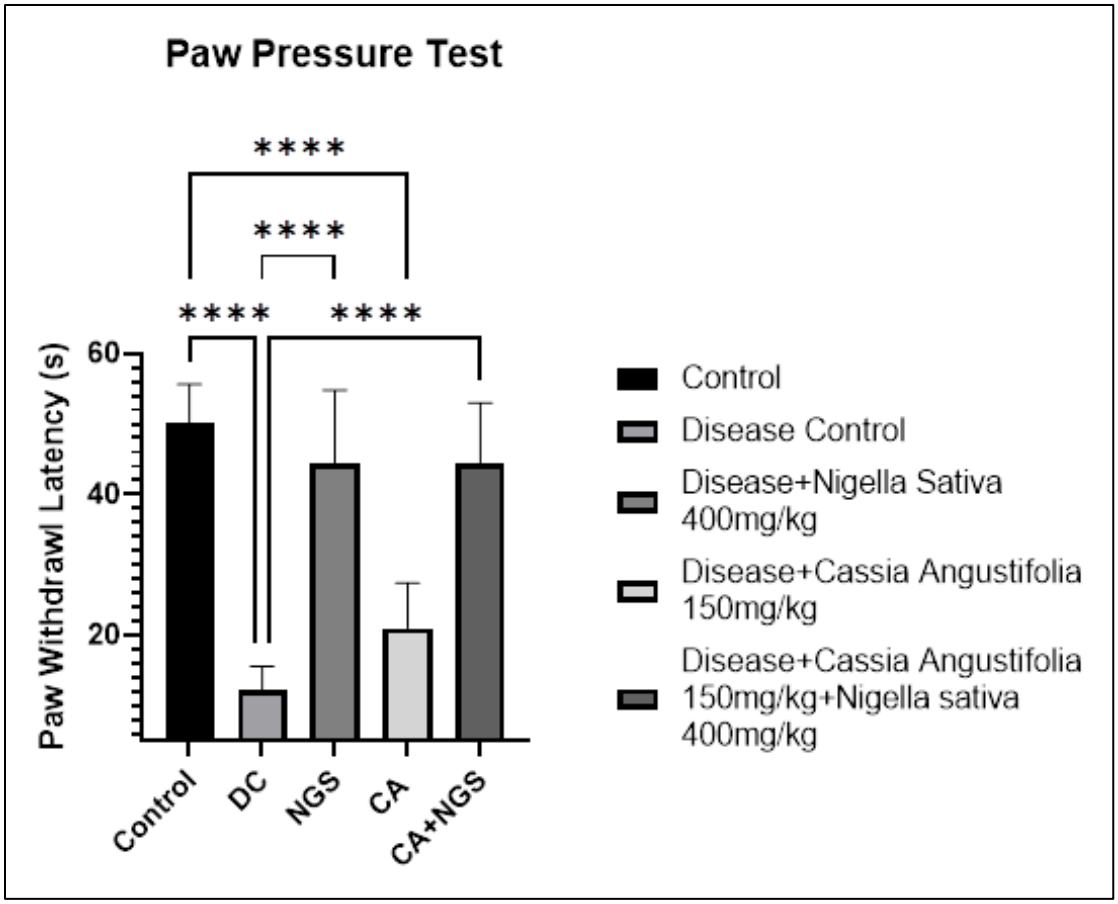

Figure 29: Paw Pressure Latency recorded. The paw pressure test experiment has produced results indicating a significant increase in the paw withdrawal threshold of the control group with the disease.

3.3.4. Tail Flick Test

Similar significant increases in tail flick latencies were seen when NS and CA+NS treated animals were contrasted with the STZ control group of mice. CA-treated mice demonstrated a slight but insignificant decrease in tail flick latency after prevention in comparison to the STZ control.

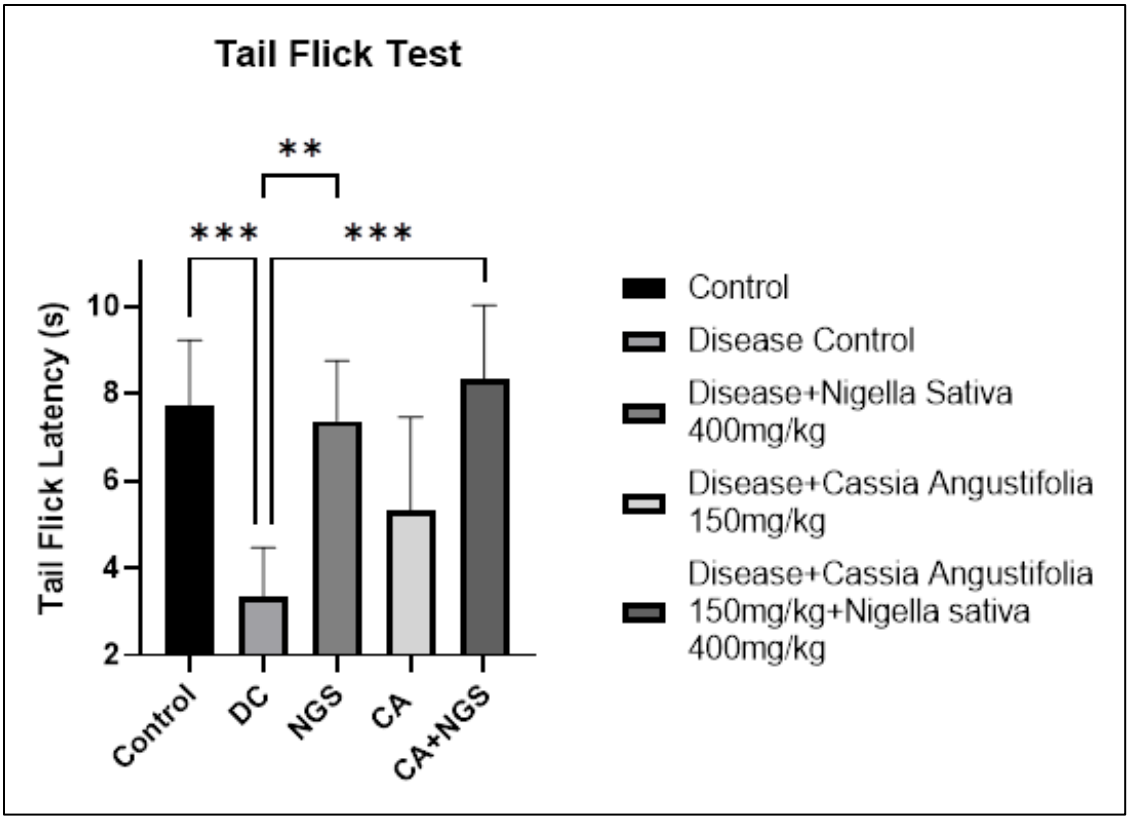

Figure 30: Tail Flick Latency recorded. The Tail Flick test has demonstrated that the tail withdrawal latency in the treatment groups was greater than that of the control group with the disease.

3.4. Histopathological Analysis

A histological analysis of the sciatic nerves in STZ-induced diabetic mice revealed weak, loose, and disordered myelin coating of the myelinated nerve fibers. Many inflammatory cells infiltrated the severed nerve fibers in the meantime. After being treated with NS, CA, and combined CA+NS extracts, only minimal myelin sheath degradation and a few scattered inflammatory cells were seen in the Preventive and Curative groups. In the automobile category, there were no clearly visible injuries. Computer-aided morphometric analyses of myelinated nerve fibers were carried out in the experimental groups.

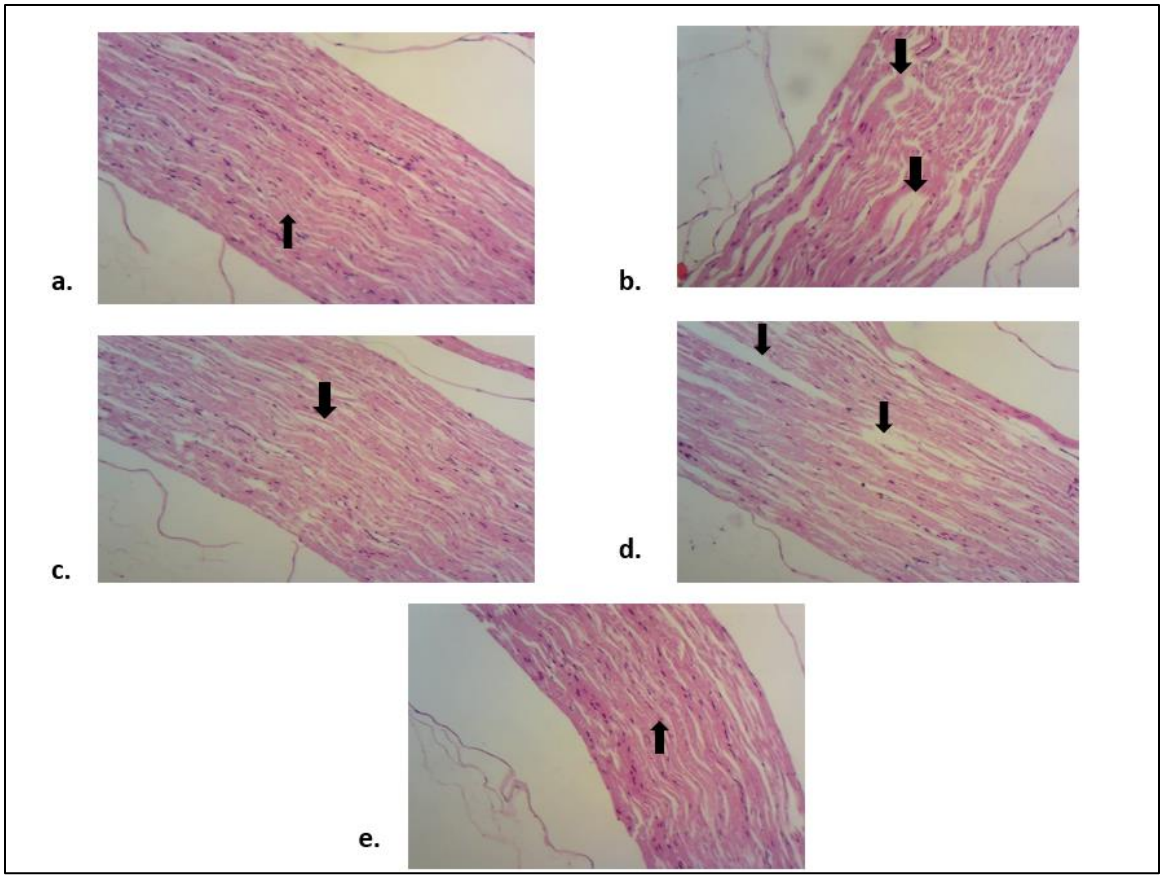

Figure 31: Sciatic Nerve tissues stained with Hematoxylin and Eosin. Microscopic pictures of mice's sciatic nerve of different experimental groups **A** control group, **B** diseased group, **C** treated with NS, **D** treated with CA, **E** treated with both extracts. Arrows represents demyelination of the nerve fibers. Myelin coating of the myelinated nerve fibers that seems weak, loose, and disordered.

3.4.1. Cell count graph

The findings unmistakably demonstrate that there are significantly less Schwann cell nuclei in ill animals than in healthy controls. There are no discernible differences in the dosages of both extracts given to the animals. When NS is administered in quantities to mice, the number of neurons is nearly normal. The total quantity of neurons in mice given doses of CA differs noticeably from control mice but not from ill mice. Mice receiving both extracts demonstrate the most notable distinction from diseased mice.

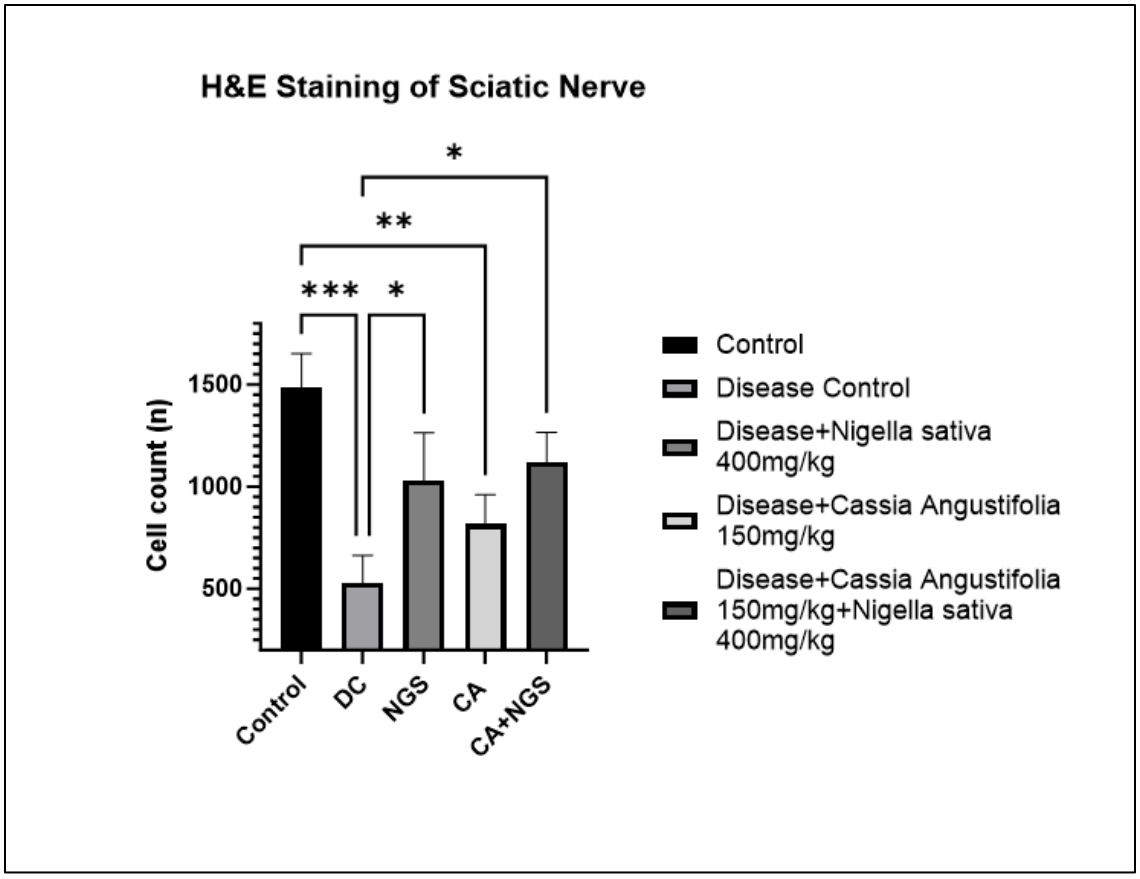

Figure 32: Schwann cell nuclei count after histopathology analysis. The present graph portrays the utmost Schwann cells nuclei's depletion in mice afflicted with disease.

3.5. Gradient PCR Optimization results

RNA extraction was followed by gel electrophoresis to evaluate the sample's purity. The outcome of optimization is depicted in the figure. Beta-actin bands were seen at 358 base pairs, while MPZ and NfL bands were seen at 70 and 68 base pairs, respectively. By analyzing the existing research, the amplicon size was determined and optimized at 62°C for MPZ AND 61°C for both beta-actin and NfL. The forward and reverse primers' operating ranges were derived from the BLAST method. The first forward primer range was deducted from the final reverse primer range.

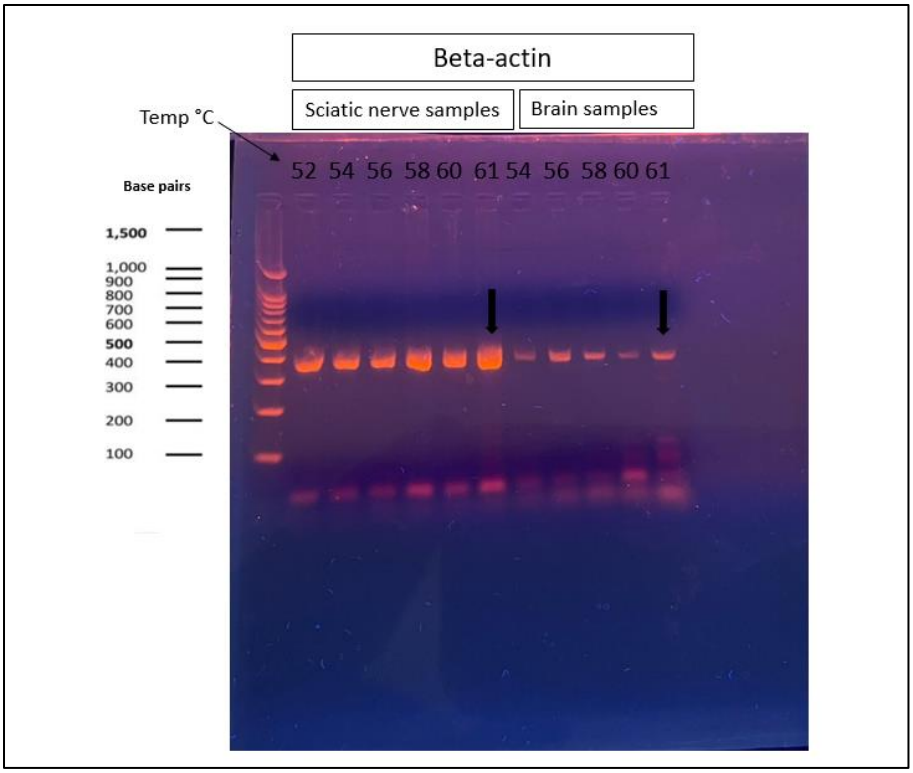

Figure 33: PCR optimization results. Gel shows dense bands of 358bp at 61°C which found out to be best annealing temperature for Beta-actin.

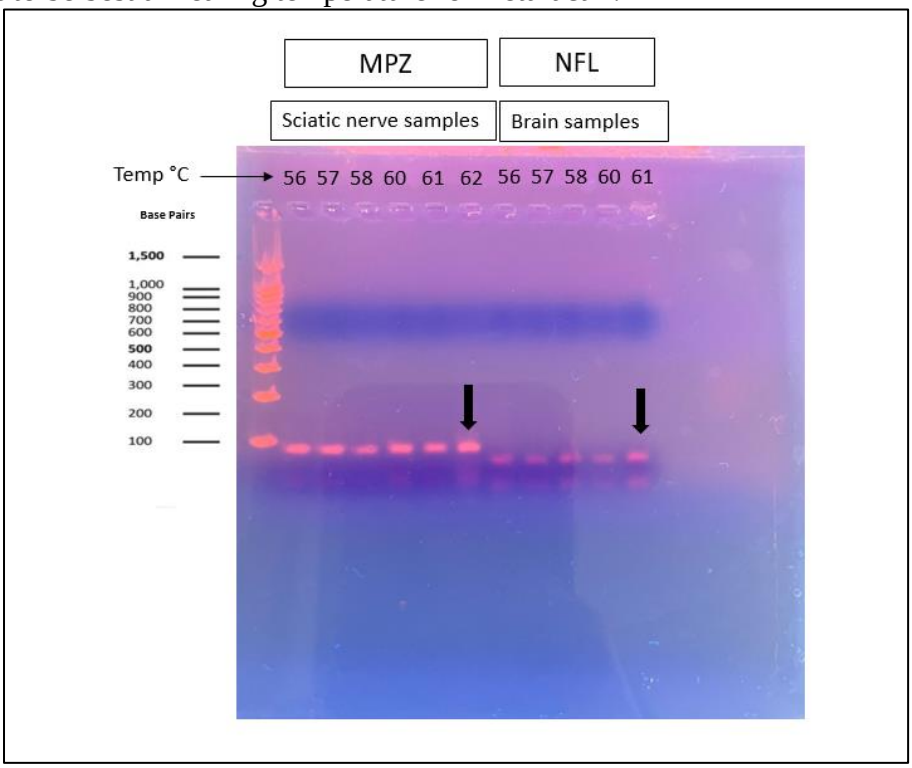

Figure 34: PCR optimization results. Gel shows dense band of 70bp at 62°C and 68bp at 61°C which found out to be best annealing temperatures for MPZ and NfL respectively.

3.6. q RT-PCR results

Analysis of the data produced during the quantitative polymerase chain reaction experiment, which showed that there was a substantial difference in the expression of both MPZ and NFL proteins in control and diabetic mice, is required to evaluate the results of qPCR. The expression of proteins has improved in the treated groups, and it is clear how much the amount of expression of both proteins has been up- and down-regulated in the various groups. In comparison to diseased groups, MPZ expression was increased in the preventive groups, whereas NfL expression was downregulated in the prevention groups. However, the delta delta CT graph depicts fold change relative expression to the housekeeping gene beta-actin or mRNA relative expression of all proteins.

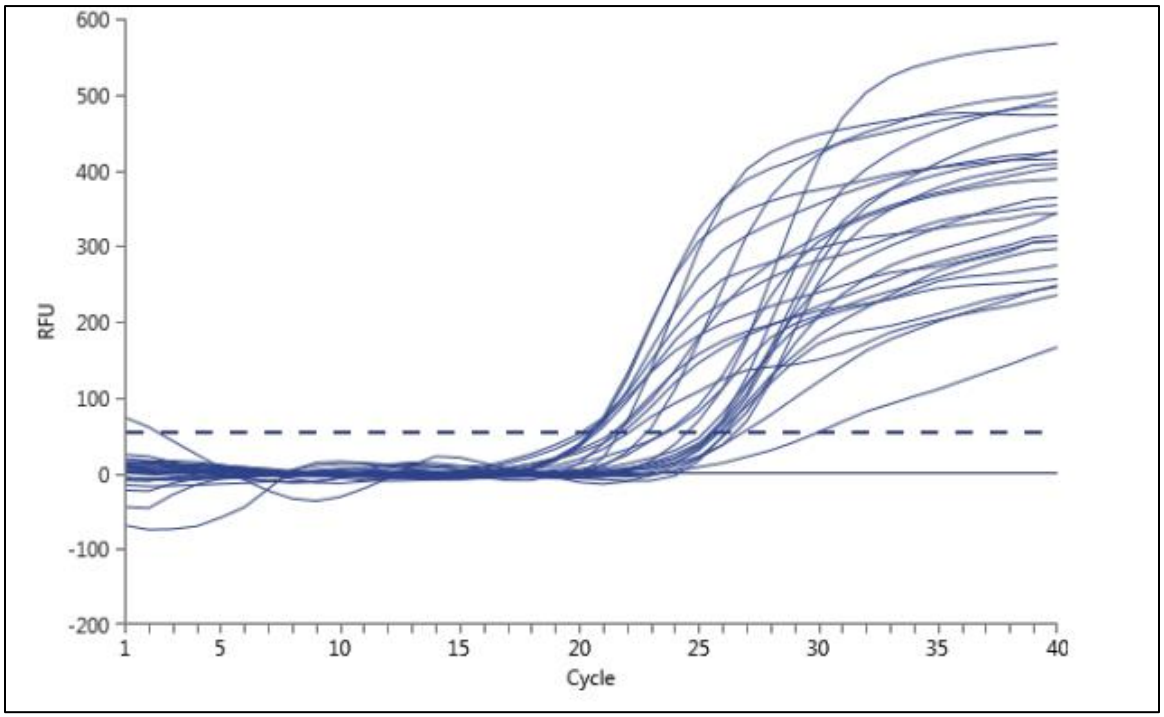

Figure 35: Representative amplification curves. This graph shows amplification curves for Beta-actin expression in q-PCR analysis

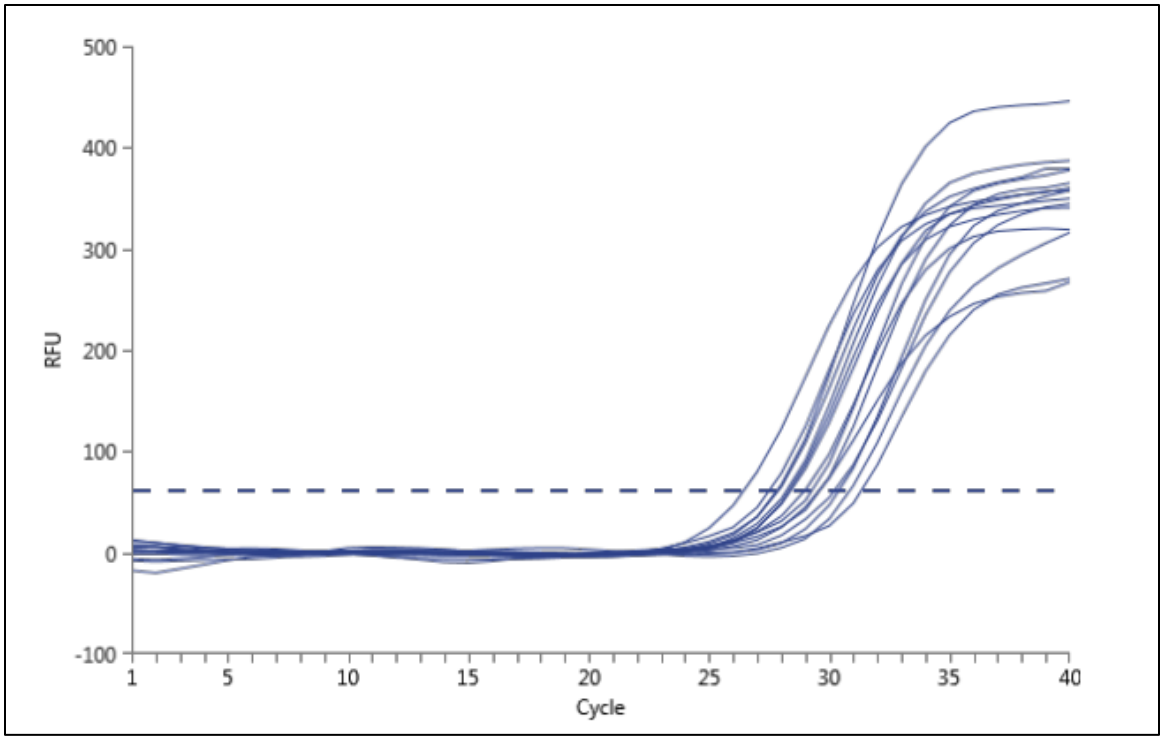

Figure 36: **Representative amplification curves.** This graph shows amplification curves for MPZ expression in q-PCR analysis

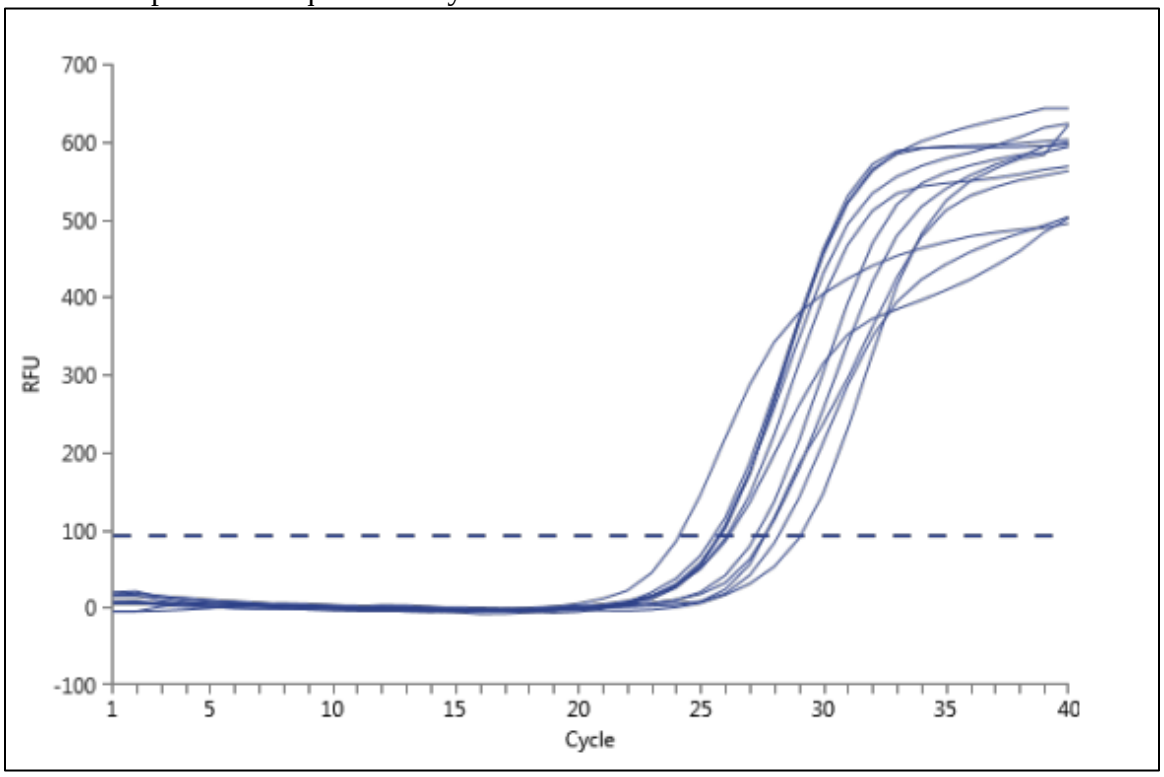

Figure 37: Representative amplification curves. This graph shows amplification curves for NfLexpression in q-PCR analysis

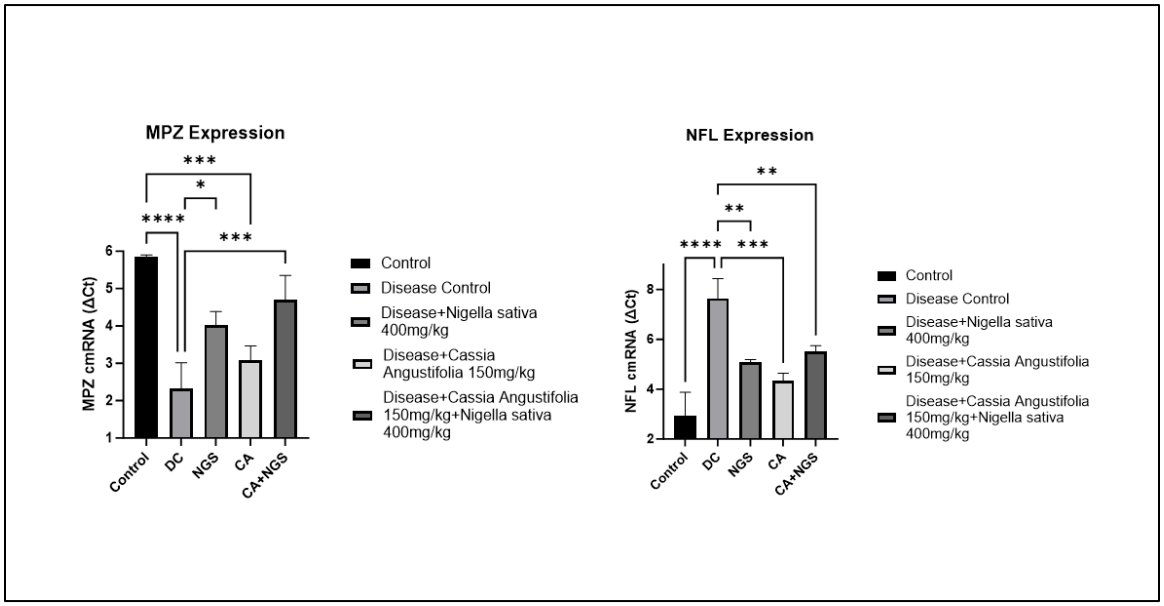

Figure 38: ΔCt normalization values after qPCR Analysis. The graphs depicts ΔCt values of all the groups after qPCR analysis. There is significant difference in both MPZ and NFL protein expression of control and diabetic mice. Treated groups have shown improvement in protein expression.

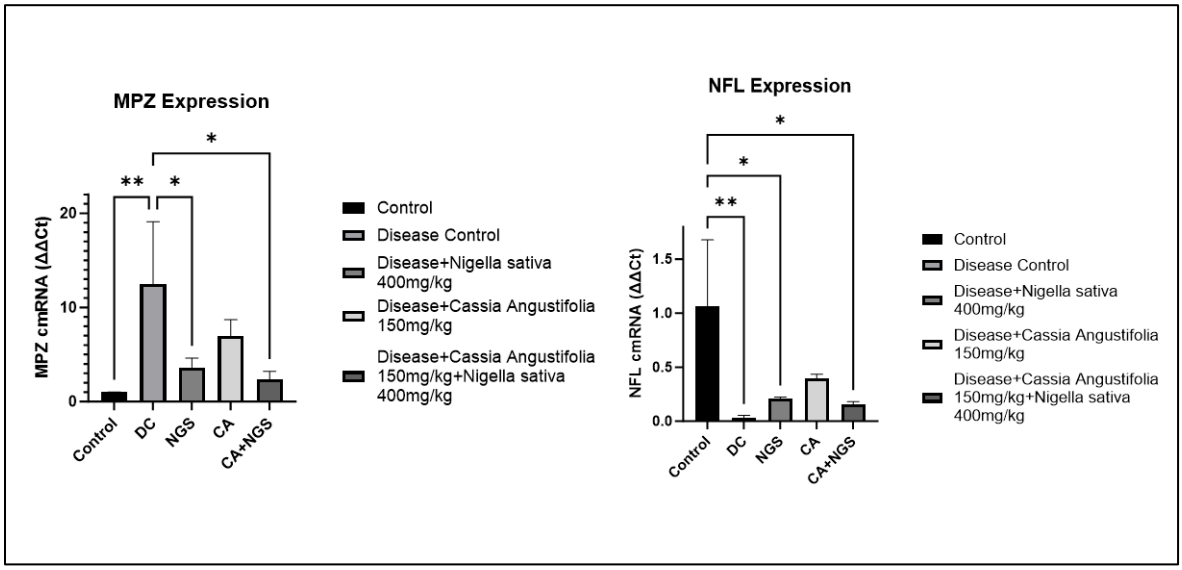

Figure 39: ΔΔCt values after qPCR Analysis. The graphs depicts ΔΔCt values of all the groups after qPCR analysis. There is significant difference in both MPZ and NFL protein expression of control and diabetic mice. Treated groups have shown improvement in protein expression and the level of up regulation and downregulation in expression of both proteins in different groups is visible.

Chapter 4
Discussion

Diabetes mellitus, commonly known as Diabetes, comprises a range of disorders with autoimmune, metabolic, and genetic origins that cause hyperglycemia (Kaveeshwar & Cornwall, 2014). The main precipitating factor for Diabetic neuropathy is prolonged exposure to elevated levels of blood glucose, characterized by neuronal damage and disruption of nerve signaling (Cameron et al., 2001). 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 focus has been directed toward the role of oxidative stress as a crucial pathophysiological mechanism in the development of Diabetic Peripheral Neuropathy (DPN). The overproduction of reactive oxygen species (ROS) and the disruption of antioxidant defense systems lead to a disturbance in redox balance, thereby promoting oxidative damage in diabetic peripheral neuropathy (DPN). These findings provide innovative therapeutic approaches for DPN that target oxidative stress (Premkumar & Pabbidi, 2013).

Recent research has established that commonly used plant chemicals possess advantageous neuroprotective, antioxidative, and anti-neuroinflammatory properties that can effectively enhance diabetic peripheral neuropathy (DPN) (Singh et al., 2014). 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. Previous studies have indicated that patients with diabetic neuropathy have a deficient endogenous opioid peptide system that is linked with heightened pain perception (Akintoye et al., 2020).

Our analysis of hyperalgesia and allodynia in mice revealed a significant disparity between control and diseased subjects. However, herbal doses were found to 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. H&E staining of sciatic nerve tissue from DPN mice indicated demyelination and dysregulated Schwann cells, while treated groups displayed improvement. Previous studies have noted that participants with diabetic neuropathy

exhibit a significant decrease in mRNA MPZ expression levels, while NfL protein levels are increased. Similarly, our q-PCR data showed a decrease in MPZ mRNA expression in a mouse model of DPN, while treatment with NS, CA, and a combination of the two led to a relative increase in mRNA expression. On the other hand, NfL mRNA expression in the DPN mouse model increased, while herbal treatment groups showed a decrease in its expression. The sciatic nerves that were exposed to STZ exhibited a more favorable response in both biochemical and morphological aspects when treated with nigella sativa therapy (Do Carmo et al., 2008).

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. A study was conducted that demonstrated that the administration of NS extract and TQ to rats with cerebral hypoperfusion led to enhancements in their learning and memory processes by reducing hippocampal oxidative stress and AChE activity. The neuroprotective activity of Cassia angustifolia 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 (Kwon et al., 2021). The aforementioned studies suggest the beneficial effects of CA and NS for neuroprotection. Our behavioral, histopathological, and molecular analysis depicts the significant role of these two herbs in preventing DPN and cognitive impairment.

The frightening rise in DN prevalence and its catastrophic effects on patients come at a high cost. More than half of people with diabetes are affected by DN, which can result in foot ulceration and Charcot's foot as well as symptoms ranging from neuropathy to instability and discomfort. Recently, it has become clear that DN has psychosocial repercussions and contributes to high rates of morbidity.

Numerous supervised exercise programs have been created, including training for endurance, balance training, sensorimotor training, gait training, vibration therapy for the entire body, resistance training, physiotherapy, and rehabilitation. All of these programs have positive effects but are challenging to implement in healthcare systems with limited resources. The best program has been suggested as a combination of endurance and

sensorimotor training; nevertheless, these programs typically have low compliance. Programs that are customized could be more appealing. For instance, it has been shown that balance training is especially effective in the treatment of psychological illnesses. Additionally, offering diabetes and diet advice may aid in weight management, glycemic control, and exercise program adherence.

To ensure the early implementation of disease preventive initiatives, screening and early detection are essential. Numerous methods of screening and evaluation for diagnosis are available, but many of them are ineffective hence miss people with early DN. Additionally, nerve conduction tests, the present technique for identifying DN (Lauria et al., 2010). Another approach, Quantitative Sensory Testing (QST), is a noninvasive diagnostic technique that assesses tiny fiber performance but is time-consuming, expensive, and demands a high level of skill (Bouhassira et al., 2007).

Innovative methods, such as corneal confocal microscopy, are under investigation and hold promise as a quick and reliable test for early DN diagnosis (Tavakoli et al., 2010). However, it is key to note that more research is necessary to strengthen the evidence supporting its regulatory clearance and widespread adoption (Pritchard et al., 2015).Patients with insensate or painful DN have few therapeutic choices. These pharmaceutical treatments have been associated with a broad spectrum of side effects and display evidence that they are moderate to low quality. Opioids can occasionally be recommended as a third-line pharmacotherapy. However, fresh advice advises against using it completely due to significant risks. Studies utilizing 8% topical capsaicin indicate effectiveness for DN despite the intermediate to low evidence for topical analgesics. In the absence of further studies, IV medications should only be administered in refractory situations.

Innovative, non-pharmacological neuromodulation therapies hold the potential to enhance nerve conductivity and pain results. SCS has just been authorized for extremely painful DN, despite the dearth of excellent proof and the related procedural risks. Despite the fact that there are many treatments and medications available to treat diabetic neuropathy, these drugs' drawbacks, including poor pharmacokinetics, high costs, and drug resistance, make it necessary to switch from chemical medications to natural

conventional medicines in the form of herbal treatment. In Indian medicine, plants have long been employed as a source for various drugs. The Rigveda provides a thorough account of all the therapeutic properties of herbal remedies. In order to treat pain brought on by nerve cells, herbal treatments were used in medieval Persia.

Pain was treated with topical herbal ointments made from hot-tempered oils like thyme, pepper, colocynth, flax, and chamomile. Lavender syrup is the most popular treatment for neuropathic pain, based on experts in medieval Persian medicine. As a complementary therapy, they also used Takmid (warm compresses) (Tremont-Lukats et al., 2000). After some time, many different synthetic drugs were created and introduced, however many were unsuccessful due to defects. Plants are regaining popularity, and people are using them confidently. Due to their efficiency, lack of side effects, and low cost, herbal medicines are frequently given, and traditional plant therapies are used all over the world for a range of diabetes conditions.

Managing neuropathic pain in individuals with diabetes mellitus is of paramount importance, given that diabetes is often referred to as an "invisible killer" due to its propensity to cause neuropathy and severe pain (Vinik et al., 2013). Synthetic drugs commonly prescribed for diabetes and neuropathic pain management may carry the risk of serious side effects and exhibit suboptimal pharmacokinetics (Mogensen et al., 2018). Consequently, there is a growing emphasis on exploring natural remedies with inherent anti-diabetic, antioxidant, and anti-inflammatory properties to address hyperglycemia and alleviate neuropathic pain (Ojo et al., 2015).

Medicinal herbs, in particular, have gained attention as potential alternatives to synthetic pharmaceuticals due to their perceived safety and efficacy (Ekor, 2014). Nevertheless, it is essential to note that the utilization of medicinal plants as therapeutic agents requires ongoing research and development. This continuous effort is crucial for maintaining the credibility and progress of herbal medicines in the market.

To achieve this, aligning herbal medicine with the principles of the Primary Healthcare Strategy (PHC) can be a productive approach. The PHC strategy emphasizes accessible, community-based healthcare that focuses on prevention, early intervention, and holistic well-being (World Health Organization, 1978). Integrating herbal medicine into this

framework can promote its responsible use, ensure patient safety, and facilitate evidence-based practices.

To maintain the usage of therapeutic herbs, efforts must be done. Data should be kept geographically in tabular form to ensure accurate information on plant occurrence in specific areas (Smith et al., 2019). Research and development should frequently be shared to prevent duplication. Standardization of medicinal plants is essential for future research and must take safety into account. When manufacturing herbal medication, the dose amount should also be taken into account since if it is too tiny, the drug will not work, and if it is excessive, the drug will be hazardous.

As some pharmaceuticals have been associated with chemical drugs, it is also crucial to discover the pharmacokinetic properties of bioactive components in order to reduce herb-drug interactions. For instance, Ginkgo biloba is a herb that has been approved for the treatment of neuropathic pain, but because it also thins the blood, it is not advised for those who take aspirin, nonsteroidal anti-inflammatory drugs, or anticoagulants (Bauer, 2012). Additionally, reliable and efficient bioassays for determining the necessary bioactive compounds should be developed. Clinical trials have not yet been carried out on the majority of anti-diabetic medications made from medicinal plants, which are typically preventive rather than therapeutic. New herbal drugs for human use could become accessible very soon if such studies are sponsored and carried effectively.

The complex and serious lifestyle disease known as diabetic neuropathy is on the rise and may soon rank among the health epidemics. Excessive sugar intake, obesity, inactivity, and environmental factors are all linked to high glucose levels, which damage the peripheral nerves in the brain and cause diabetic neuropathy. Its development is significantly influenced by other lifestyle factors as well as a dearth of understanding of nutritional practices. The currently available medications are hardly ever utilized because of their poor pharmacokinetics and negative effects.

If efforts to use medicinal plants are made in the future, DN may be treated with little to no side effects thanks to herbal medicinal herbs and their bioactive components that are involved in analgesic effects and pathways like neuroprotective, anti-inflammatory, anti-apoptotic, anti-oxidant, and calcium-inhibitory actions. Although the useful effects of

plants in the treatment of DN have been mentioned, clinical trials have not yet been investigated. As was previously said, in order to avoid the issues associated with synthetic pharmaceuticals, herbal products should be encouraged and pushed (Vinik et al., 2013).

Conclusions

We worked on DPN associated with TIDM in mouse model. We obtained promising results by treated TIDM mice by natural herbs. The brain activity of treated mice was enhanced by these herbs. We concluded that their further research is warranted to bring these alternative medicine from bench to bedside. There is a hope of cure for diabetic neuropathy in these herbal medicines. Moreover, herbal medicines have less side effects as compared to traditional allopathic medicines.

Supplementary

Delta CT method for qPCR

Groups	Sample	Ct (MPZ) LOW IN DPN	Ct (B-ACTIN)	ΔCt	ΔΔCt Avg= 5.842	2^-ΔΔCt
Control	A1	28.355	22.879	5.773	-0.069	1.04
	A2	27.848	23.621	5.876	0.034	0.97
	A3	30.434	24.556	5.878	0.036	0.97
DC	B1	26.480	23.746	2.734	-3.108	8.62
	B2	28.988	26.230	2.758	-3.084	8.47
	B3	29.316	23.440	1.487	-4.355	20.4
NS	C1	27.543	26.056	4.227	-1.615	3.06
	C2	30.871	26.629	4.242	-1.6	3.03
	C3	29.254	25.684	3.57	-2.272	4.82
CA	D1	27.902	24.980	2.922	-2.92	7.56
	D2	29.654	26.121	3.533	-2.309	4.95
	D3	28.434	25.660	2.774	-3.068	8.38
CA+NS	E1	31.363	25.590	5.476	-0.366	1.28
	E2	29.605	25.301	4.304	-1.538	2.90
	E3	30.246	25.895	4.351	-1.491	2.81
Groups	**Sample**	**Ct (NFL) HIGH IN DPN**	**Ct (B-ACTIN)**	**ΔCt**	**ΔΔCt Geometric Avg= 2.847**	**2^-ΔΔCt**
Control	A1	24.129	20.168	3.961	1.114	0.46
	A2	25.801	23.746	2.055	-0.792	1.73
	A3	29.066	26.230	2.836	-0.011	1.00
DC	B1	27.629	20.660	6.969	4.122	0.05
	B2	30.059	21.496	8.563	5.716	0.01

	B3	28.176	20.785	7.391	4.544	0.04
NS	C1	25.566	20.629	4.937	2.09	0.23
	C2	26.902	21.762	5.14	2.293	0.20
	C3	27.262	22.121	5.141	2.294	0.20
CA	D1	25.848	21.824	4.024	1.177	0.44
	D2	25.754	21.098	4.656	1.809	0.28
	D3	25.832	21.512	4.32	1.473	0.36
CA+NS	E1	26.066	20.402	5.664	2.817	0.14
	E2	27.645	22.440	5.205	2.358	0.19
	E3	26.160	20.543	5.617	2.77	0.14

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