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SU92-MSZGW-F23-017

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**INVESTIGATION OF NEUROPROTECTIVE EFFECTS OF *Ziziphus jujuba*
FRUIT EXTRACT IN PARKINSON'S DISEASE**



SUPERIOR UNIVERSITY

Thesis Submitted to

The Superior University Lahore

In Partial Fulfillment of the

Requirement for the Degree of

MS Zoology

By

QANDEEL NASIR

Roll No. SU92- MSZGW-F23-017

Session: 2023-2025

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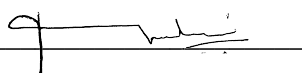
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This is to certify that the research work presented in this thesis, titled **“Investigation of Neuroprotective Effects of *Ziziphus jujuba* Fruit Extract in Parkinson’s Disease”** was conducted by **“Qandeel Nasir”** under the supervision of **“Dr. Tahir Mehmood”** and Co-Supervision of **“Dr. Chand Raza”**.

No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Faculty of Sciences, The Superior University, Lahore in partial fulfillment of the requirements for the degree of Master of Science in the field of **“Zoology”** in Faculty of Sciences at The Superior University, Lahore.

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Dedication

I dedicate my research to the **Almighty Allah**, for his guidance, and wisdom, and for providing me with a healthy life to carry out this research work. All of these I offer to you.

I wholeheartedly dedicate this thesis to my beloved parents, **Nasir Ali Mughal** and **Rubina Nasir** whose endless love, unwavering support, and countless sacrifices have been the foundation of my strength and success. Their prayers and encouragement have been my guiding light throughout every step of this academic journey.

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List of Abbreviations

[Abbreviations not explained in the text]

PD	Parkinson's disease
SNCA	Synuclein Alpha
LRRK2	Leucine rich repeated kinase 2
PINK1	PTEN induced kinase 1
PARK7	Parkinsonism Associated Deglycase
DAT	Dopamine Active Transporter
ROS	Reactive Oxygen Species
ATP	Adenosine Triphosphate
SNPc	Substantia Nigra Pars Compacta
SOD	Superoxide Dismutase
MAO	Monoamine oxidase A

ABSTRACT

Parkinson's disease, the second most extensive neuroprogressive illness, is marked by a gradual declining of dopamine producing neurons in the substantia nigra pars compacta. In addition to non-motor symptoms like mood fluctuations and memory loss, this condition causes severe motor dysfunctions like bradykinesia, muscle rigidity, and tremors. The pathological features of PD include oxidative stress, protein aggregation, apoptotic death of neurons, and dysfunctioning of the mitochondria. Since current pharmaceutical treatments, like levodopa and dopamine agonists, only relieve symptoms and do not reverse the progression of the disease, an essential need is required to research new modalities based on natural sources. *Zizyphus jujuba*, also known as jujube or red date, is a well-liked medicinal fruit that is abundant in bioactive compounds like polysaccharides, alkaloids, flavonoids, and saponins. It has been found that such compounds possess antioxidant, anti-inflammatory, and anti-apoptotic qualities. These properties generated the idea that *Z. jujuba* provided neurological protection against rotenone-induced Parkinsonism. The ethanolic extracts of *Z. jujuba* fruits prepared for the current study and subjected to phytochemical profiling using Gas Chromatography–Mass Spectrometry contained neuroactive metabolites such as flavonoids, alkaloids, fatty acids, and saponins. Adult mice were divided into six groups: control, rotenone-treated, three *Z. jujuba* extract-treated groups with different concentration of dose and Sinemet treated group. PD was induced by chronic exposure to the mitochondrial complex I inhibitor rotenone; the extract-treated group was also administered ethanolic fruit extract. Behavioral performance was assessed using locomotor and motor coordination tests. Anti-oxidative biomarkers were analyzed by gene profiling. In addition, histological examinations of the substantia nigra and striatum were conducted to assess the neuronal integrity.

The results demonstrated significant dopaminergic neuronal degeneration, increased oxidative stress, and significantly decreased motor function following rotenone treatment. The administrations of *Z. jujuba* extract improved locomotor behavior and motor coordination compared to the rotenone group. According to histological analysis, the group that received the extract treatment had lesser neuronal damage and retained neuronal architecture. GC-MS profiling showed the presence of

neuroprotective compounds, including rutin, kaempferol, and derivatives of oleanolic acid, which may account for the pharmacological effects that were observed. In conclusion, the outcomes of this study provide strong experimental evidence that an ethanolic extract of *Zizyphus jujuba* fruit considerably preserves neurons in a rotenone-induced PD model in mice. The extract preserved dopaminergic neuronal survival, bolstered antioxidant defense systems, and improved behavioral deficiencies. These findings indicate that *Z. jujuba* may be useful as a natural neuroprotective agent, but further study is required to explain its molecular pathway of action and clinical applicability for Parkinson's disease treatment.

CHAPTER NUMBER 1

Introduction

1.1: Parkinson's disease (PD)

Parkinson's disease is a neuroprogressive condition which causes dopamine-producing cells, or dopaminergic neurons, in the substantia nigra pars compacta region of the midbrain to die. It results in a reduction of motor function. James Parkinson first described "shaking palsy" in 1817. Today, PD is regarded as the second most prevalent age-related neuroprogressive disorder after Alzheimer's disease, and its occurrence is rising as the world's population ages [1]. Over 10 million people around the world are supposed to be impacted by PD and by 2040, that number is predicted to increase to over 20 million (Dorsey, Sherer, Okun, & Bloem, 2018). Although some people can exhibit early onset Parkinson's symptoms before the age of 50, PD is more common in those over 60. The current ratio of men to women diagnosed with the condition is 1.5:1, meaning that men are diagnosed with it more often than women [2]. About 1% to 2% of people over 65 have Parkinson's disease, which typically manifests between the ages of 55 and 65. The percentage rises to 3.5% by the ages of 85-89 after the age of 60 [3, 4]. It affects about 0.3% of the population [5]. The risk of contracting and progressing is known to be influenced by both genetic and environmental factors.

Even so the precise causation PD remain uncertain, it is supposed to be a combining effect of environmental and genetic factors. SNCA, LRRK2, PINK1, PARK7, and PRKN mutations account for up to 5–10% of familial PD cases [6]. The dysfunction brought on by oxidative stress, misfolded proteins, and autophagy impairs the mitochondria linked to these genes and is a crucial parameter in the development of PD. Living in a rural area, drinking a lot of well water, being around pesticides like rotenone and paraquat for an extended period of time, and being around heavy metals are all environmental risk factors [7].

A naturally occurring substance called rotenone is extracted from the roots and stems of some plants, especially those belonging to the Derris and Lonchocarpus species. It has long been used as a pesticide, insecticide, and piscicide. Because of its unique chemical makeup (lipophilic fused-ring system and methoxy groups), rotenone is

very good at getting past the blood-brain barrier (BBB) and building up in the mitochondria of neurons [8]. The chemical structure of rotenone is shown in (Fig: 1.1). By blocking mitochondrial complex I, producing oxidative stress, and encouraging neurodegenerative alterations like α -synuclein aggregation, it specifically damages dopamine producing neurons in the substantia nigra [9]. This makes it an effective tool for understanding the pathology of PD and simulating the condition in animals.

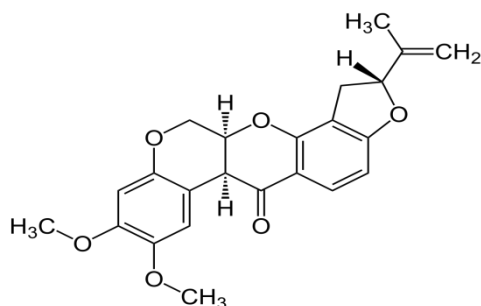


Figure 1.1: Chemical structure of rotenone

Aberrant forms of α -synuclein are linked to PD and may be the determinant to cell death from both environmental and genetic factors [10, 11]. On the other hand, although the reasons for this are still unclear, smoking and caffeine use have been connected to a decreased chances of developing PD [12].

Parkinson's disease can be categorized as late (beyond 60 years old), early (21–50 years old), or juvenile (under 21 years old). The juvenile variant is rare, atypically presented, familial (up to 50% of cases), and related with a mutation in the parkin gene [13, 14]. 10% to 16% of people have PD, and those who have first- or second-degree relatives are more susceptible to get it than the general population [14, 15]. Parkinson's disease with an early or late onset has equal chances to have a positive family history [16].

1.1.1: Pathophysiology

Parkinson's disease is principally marked by the selective decline of dopamine producing neurons in the SNpc. This results in a major drop of dopamine levels in

the striatum, a part of the brain that is closely related to the regulation of voluntary movement (Fig 1.2). According to pathologic research, Parkinson's disease symptoms are specific to each affected area and are resulted due to the gradual degeneration of neurons over time. The basal ganglia circuitry that initiates and regulates motor functions is hampered by the loss of these neurons [17].

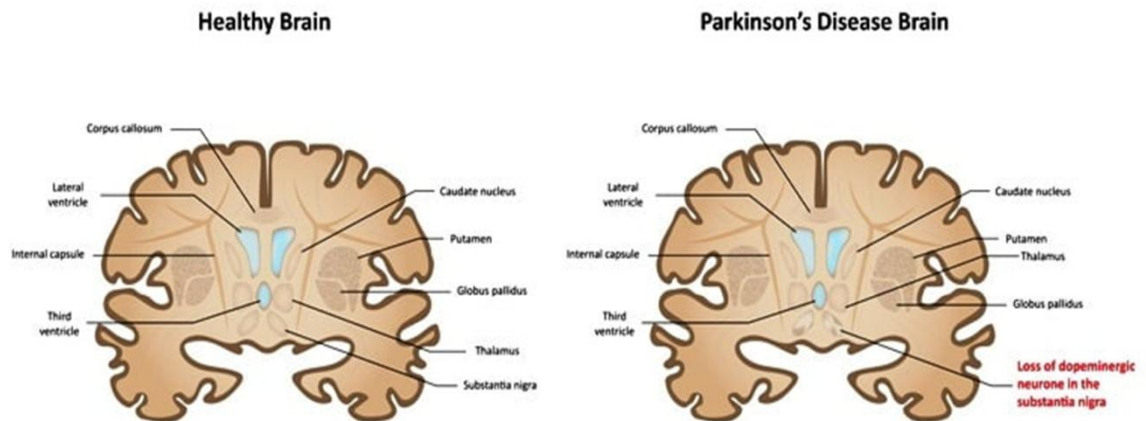


Figure 1.2: Pathophysiology: Left side shows healthy brain. Right side shows diseased brain.

Lewy bodies, also referred to as inclusion bodies, are another distinguishing feature of Parkinson's disease. Alpha-synuclein protein aggregates that are abnormally folded and found inside cells make up these bodies. These bodies are thought to be pathognomonic of Parkinson's disease and are usually present in the remaining neurons [18]. The Braak staging theory of Parkinson's disease states that α -synuclein pathology involves particular brain areas and develops in a predictable order. Lewy body deposition first manifests in the olfactory structures and the dorsal motor nucleus of the vagus nerve in medulla (Stage I). Parkinson's disease's characteristic motor symptoms appear in Stage II and III when the disorder spreads to the midbrain's substantia nigra, locus coeruleus, and subcoeruleus complex. Cortical involvement, especially in the temporal, frontal, and other neocortical areas, is finally seen in Stage IV when it is linked to cognitive and neuropsychiatric disorders [19]. The Braak staging of Lewy body deposition is described in (Table 1.1). Both the motor and non-motor symptoms of PD are considered to be caused by the

progressive development of alpha-synuclein pathology in non-motor regions of the brain [20].

When motor symptoms appear, a pathologic analysis of the substantia nigra shows 30–70% cell loss [21]. The main objective of treatment is to control the damaged circuit and replace dopamine with dopaminergic medications. Cognitive impairment, mood disorders, and problems with impulse control can result from deficits in serotonergic and noradrenergic systems or dopamine outside of the basal ganglia [22, 23].

Table 1.1: Braak Staging of Lewy Body Deposition

Stage	Sites Affected by Lewy Body	Major Symptoms
I	Dorsal motor nucleus of the vagus nerve and olfactory tract	Constipation, anosmia
II	Locus coeruleus and subcoeruleus complex	Constipation, anosmia
III	Substantia nigra	Motor symptoms of Parkinson disease
IV	Cortical involvement	Dementia, psychosis

1.1.2: Clinical Features

Both motor and non-motor symptoms are present in Parkinson's disease. The major motor signs are Bradykinesia (slowness of movement), Rigidity (increased muscle tone), Resting tremor, typically asymmetric and appearing in one limb Postural instability, which frequently appears in later stages [24].

Non-motor symptoms, which can appear years before motor symptoms do, are becoming more widely acknowledged for their major effect on overall quality of life. These consist of Sleep disturbances, Autonomic dysfunction (constipation, orthostatic hypotension), olfactory dysfunction, depression and anxiety [25].

1.1.3: Diagnosis

The majority of PD diagnoses are made clinically based on a neurological examination and medical history. The Movement Disorder Society's (MDS) Clinical Diagnostic Criteria for Parkinson's disease and the UK Clinical Diagnostic Standards of the Parkinson's Disease Society Brain Bank [26]. Neuroimaging methods such as dopamine transporter (DAT) SPECT scans, which reveal dopaminergic deficiencies in the striatum, can aid in the diagnosis even though they are not always used. There is currently no reliable biomarker for PD diagnosis, despite efforts to identify molecular, genetic, or imaging-based biomarkers that could aid in an early and precise diagnosis [27].

The disorder's motor symptoms, which frequently start asymmetrically, include small handwriting (micrographia), masked facies (which initially manifests as a decreased blink rate), a soft voice (hypophonia), resting tremor, balance issues, slowness of movements, stuttering steps, and stiffness. The classic symptom of resting tremor, which usually affects one upper limb, may not be present in 20% of patients [24]. Thirty percent of patients may initially present with lower extremity tremor in addition to a resting lip, jaw, or even tongue tremor [24, 28].

Essential tremor should be considered in the differential diagnosis because head and speech tremors are uncommon [24]. Among all these traits, bradykinesia has the strongest association with the level of dopamine deficiency [29]. The diagnosis is formalized by the UK Parkinson's Disease Society Brain Bank and can be made with up to 90% accuracy. Parkinson disease is also characterized by a wide range of non-motor symptoms, some of which may manifest early or late, while others may manifest before the diagnosis is established based on motor signs. The frequency of early non-motor symptoms that may manifest before Parkinson disease is diagnosed, including constipation, olfactory impairment, depression, and issues with rapid eye movement sleep behavior.

Parkinson disease patients with early stages have more noticeable stiffness and muscle weakness compared to those with late development, although they are less likely to have locomotor disruption as the initial symptom [30, 31]. Although 41% of patients with early development of PD and 63% of patients with late progression of disease presented with resting tremor in one study [30], additional research has not

consistently found a difference in the onset of tremor between patients with early and late development of PD [16]. Patients with early development of disease have longer survival times, delayed fall onset, and slower disease progression [16].

1.2: Oxidative Stress and Neurodegeneration in Parkinson's disease

While the etiology of PD is multifactorial, oxidative stress plays a key role in the cascade role to neuronal death. Oxidative stress is characterized by an imbalance between the production of reactive oxygen species and the biological system's capacity to either repair the damage or detoxifying the reactive intermediates. Excess ROS can damage cellular components such as lipids, DNA and proteins causing mitochondrial malfunction and finally, cell death [32].

One of the many causes of ROS in PD is mitochondrial dysfunctioning, especially complex I of the ETC [33]. Autooxidation and MAO action are involved in dopamine metabolism. Neuroinflammation results from the release of pro-inflammatory cytokines brought on by microglia activation. Through Fenton reactions, the substantia nigra's high iron levels encourage the production of ROS. Mitochondria are both the principal generator and target of ROS. In PD, suppression of complex I of the respiratory chain lowers ATP generation and increases electron leakage, creating superoxide radicals. Postmortem examinations have revealed reduced the activity of complex I in the SNpc of Parkinson's patients [34]. Dopaminergic neurons are uniquely vulnerable to oxidative damage due to high oxidative load from dopamine metabolism, poor antioxidant defense systems and elevated iron content in the SNpc. Dopamine undergoes enzymatic and non-enzymatic degradation, producing hydrogen peroxide and dopamine quinones, both of which are cytotoxic and contribute to protein misfolding and neuronal apoptosis [5]. Iron accumulation in the SNpc of PD patients enhances ROS generation via the Fenton reaction. This results in lipid peroxidation, damaging neuronal membranes and causes neurodegeneration [36].

In healthy neurons, antioxidant enzymes like superoxide dismutase, catalase, and glutathione peroxidase, heamoxigenase 1, thioredoxin reductase 1 and nuclear factor erythroid 2-related factor 2 neutralize ROS. However, in PD, these systems are often compromised. A major loss in glutathione levels in the SNpc is one of the earliest

biochemical changes observed [37]. Activated microglia in the PD brain release ROS, nitric oxide, and inflammatory cytokines (e.g., TNF- α , IL-1 β), creating a vicious cycle of oxidative stress and neuroinflammation that accelerates neurodegeneration [38]. Antioxidant therapies have been investigated because of the strong correlation between oxidative stress and Parkinson's disease. Although they did not perform well in extensive research, coenzyme Q10 and creatine demonstrated some neuroprotective potential in early trials. The potential neuroprotective benefits of natural antioxidants such as polyphenols, vitamin E, and plant extracts are being studied. New tactics include gene therapy methods that target antioxidant enzymes [39].

1.3: Current Treatments

Parkinson's disease cannot be cured; instead, current treatments aim to improve quality of life and lessen symptoms. Levodopa is widely used and effective medication for PD. In the brain, levodopa is transformed into dopamine, replenishing depleted dopamine stores and easing motor symptoms. Long-term use, however, has been connected to wear-off phenomena and motor issues like dyskinesias [40]. In order to enhance the effects of levodopa or delay its use in younger people, other pharmacological options include dopamine agonists (e.g., pramipexole, ropinirole), MAO-B inhibitors (e.g., selegiline, rasagiline), and COMT inhibitors (e.g., entacapone).

Essential non-pharmacological therapies include occupational therapy, speech therapy, and physical therapy. Deep brain stimulation, which targets particular brain regions like the globus pallidus internus or subthalamic nucleus, is used to modify aberrant neural activity in patients with severe PD [41]. Although neuroprotective tactics, disease-modifying medications, and stem cell therapies are also the subject of recent research, none of them have yet to show conclusive clinical trial efficacy.

1.4: Herbal Medicine and Neuroprotection

Neurological disorders such as stroke, AD, and PD are some leading reasons of disability and deaths in all around the world. Neurodegeneration is caused by intricate pathogenic processes such as oxidative stress, neuroinflammation, dysfunctioning of mitochondria, and neuronal apoptosis. Pharmaceutical treatments

used today typically have major side effects and only momentarily reduce symptoms without halting or reversing the neurodegenerative process [6]. It results in an increase in use of alternative treatment methodology, particularly those derived from traditional medical systems. Because of its multi-targeted mechanisms of action and generally low toxicity profiles, herbal medicine has become a promising source of neuroprotective medications.

Herbal medicine has long been used in ancient medical systems such as Ayurveda, Unani medicine, and Ancient Chinese Medicine. Plants have long been recognized for their therapeutic potential, which include enhancing cognitive function, reducing anxiety, and preventing age-related cognitive decline (Bhattacharya, 2011). Nearly 80% of people worldwide use traditional medicine, with herbal medicines making up a significant portion of this practice, according to the WHO [42]. The neuroprotective properties of medicinal plants are mainly due to the bioactive phytochemicals they contain, such as flavonoids, alkaloids, terpenoids, saponins, glycosides, and phenolic compounds. There are several ways in which these phytochemicals protect neurons:

Oxidative stress is one of the primary reason of neurodegeneration. Resveratrol, curcumin, and quercetin are examples of polyphenol-rich herbal extracts that can scavenge free radicals, boost the activity of endogenous antioxidant enzymes like catalase and superoxide dismutase, and stop lipid peroxidation [43]. Prolonged neuroinflammation is a hallmark of many neuroprogressive disorders. Herbal compounds such as ginsenosides from *Panax ginseng* and curcumin from *Curcuma longa* have been shown to inhibit the activation of pro-inflammatory cytokines like $\text{TNF-}\alpha$ and $\text{IL-1}\beta$, as well as $\text{NF-}\kappa\text{B}$ signaling pathways and microglia. Many compounds derived from plants affect neurotransmitter levels. For instance, PD is treated in traditional medicine with *Mucuna pruriens* alkaloids, which contain L-DOPA, the precursor of dopamine. Other herbs, like *Bacopa monnieri*, improve cholinergic activity and cognitive function. Mitochondrial dysfunction is another significant factor in neuronal death. Examples of substances that maintain mitochondrial integrity, reduce cytochrome c release, and modify apoptotic markers such as caspase-3 and Bcl-2 include barberry and baicalin [44].

The possible neuroprotective properties of a number of medicinal plants in neuroprogressive illnesses, such as PD and AD, have been investigated in recent

years. These herbal remedies work via a number of channels, including adaptogenic, anti-inflammatory, anti-oxidative, and anti-apoptotic ones. The active ingredients, modes of action, therapeutic significance, and supporting references of a few noteworthy neuroprotective plants are included in Table (1.2) [45-49].

Table 1.4: Herbal medicines and neuroprotective potential

Herbal Medicine	Bioactive Constituents	Pharmacological Actions	Neuroprotective Mechanism	Therapeutic Implication
Ginkgo biloba	Terpenoids, Flavonoids	Improves memory, enhances cerebral blood flow	Antioxidant, anti-apoptotic, improves cerebral perfusion	Protective effects in cognitive decline and dementia
Withania somnifera	Withanolides, Alkaloids	Reduces corticosterone, promotes neurogenesis	Antioxidant, adaptogenic, neurogenic	Stress-induced neuronal damage; neurodegenerative models
Curcuma longa	Curcumin	Anti-inflammatory, anti-amyloid, antioxidant	Inhibits neuroinflammation, reduces amyloid aggregation	Demonstrated promise in Alzheimer's and Parkinson's models
<i>Zizyphus jujuba</i>	Flavonoids, Saponins	Sedative, anxiolytic	Antioxidant, anti-apoptotic pathways	Potential neuroprotection in stress and PD-related damage
Mucuna pruriens	L-DOPA, Alkaloids	Improves motor function, dopamine replacement	Antioxidant, neuroprotective beyond dopamine	Used in Parkinson's treatment in Ayurvedic medicine

1.5: Challenges and Future Prospects

Although there are still many challenges to be addressed, herbal remedies show promise in neuroprotection. Differences in plant species, geographical origin, and extraction methods result in variations in active component concentration, which hinder reproducibility. Excessive dosages of some herbal substances can be harmful or interact with prescription drugs. Most of the data comes from animal and in vitro studies. Extensive clinical trials are needed to confirm safety and efficacy in humans. Nonetheless, advances in molecular pharmacology, systems biology, and phytochemical profiling (e.g., GC-MS, LC-MS) are simplifying the understanding of herbal remedies and their intricate relationships with biological systems. Combining ancient knowledge with modern scientific techniques may greatly aid in the discovery of new neuroprotective medications.

1.6: Importance and Traditional Use of *Ziziphus jujuba*

Ziziphus jujuba Mill is a *Ziziphus* species that is a member of the Rhamnaceae family of buckthorns. It is frequently called red date, Chinese date, or jujube. Throughout Asia, particularly in Korea, Pakistan, India, and the Middle East, this deciduous tree or shrub grows (Fig. 1.3). The plant has gained a lot of attention because of its nutritional value and therapeutic efficacy, which makes it a mainstay of both traditional and modern medicine.



Figure 1.3: *Ziziphus jujuba* Fruit

Ziziphus jujuba has long been used in traditional medical systems such as Ayurveda, Unani, and Traditional Chinese Medicine (TCM) due to its numerous pharmacological qualities. Its fruits, seeds, bark, and leaves are all used medicinally in various ways. According to traditional Chinese medicine, the fruit, called "da

zao," has been used for its tonic, sedative, and digestive qualities since the Shennong Bencao Jing (Divine Farmer's Materia Medica Classic, circa 200 CE). It is frequently used to nourish blood, soothe the spirit, and tonify the stomach and spleen [50]. The dried fruits are frequently added to teas, soups, and herbal concoctions.

Jujube is referred to as "Badari" in the Indian traditional medical system, Ayurveda, and is used to treat a number of conditions, such as fever, wounds, ulcers, and digestive problems. The plant is thought to have rasayana qualities, which means it encourages vigor and longevity [51]. According to Unani medicine, the fruit is used to treat blood disorders, biliousness, and pulmonary ailments as a cooling, cardiogenic, and expectorant [52].

Ziziphus jujuba fruit is rich in essential elements like vitamin C, B-complex vitamins, phenolic acids, flavonoids, triterpenic acids, and minerals like potassium, calcium, and iron. Its neuroprotective, anti-inflammatory, anticancer, antidiabetic, and antioxidant properties are supported by these components [53, 54]. Because of their sedative and hypnotic qualities, *Z. jujuba* seeds have long been used to treat anxiety, palpitations, and insomnia. Modern pharmacological studies have confirmed the GABAergic action of jujube seed extracts, confirming their traditional use as a natural sedative [55].

Jujube fruit is a popular food item and a cultural symbol of fertility and prosperity in addition to its medicinal uses in China. It is frequently incorporated into festivals and wedding customs. In Korea this fruit is a main component of traditional medicinal soups and daechucha, or jujube tea. The fruit is used in ancient medicine to treat gastrointestinal and respiratory issues and is eaten either fresh or dried in Pakistan and India [56]. In Middle East and North Africa the dried fruit is used in traditional medicine to cure constipation, colds, and general exhaustion.

Due to its increasing demand in the pharmaceutical and nutraceutical industries, *Ziziphus jujuba* is now widely grown outside of its natural habitat. Because of its potential for immunological regulation, neuroprotection, and anti-cancer effects, its bioactive ingredients such as jujubosides, betulinic acid, and caffeic acid have attracted more scientific attention [54].

Additionally, the fruit can be used in diabetic-friendly diets and functional foods due to its high fiber content and low glycemic index. According to studies it is hypothesized that the ethanolic extract of *Zizyphus jujuba* may have neuroprotective effects against Parkinsonism caused by rotenone. This is because the plant is rich in bioactive compounds like flavonoids, saponins, and alkaloids that have anti-inflammatory, anti-apoptotic, and antioxidant properties, and because oxidative stress and mitochondrial breakdown are known to play a role in the pathophysiology of Parkinson's disease. The extract is expected to enhance endogenous antioxidant defense, reduce neuronal death, and preserve motor function as a natural treatment option for PD.

Aim and Objectives

The aim of this study is to explore the neuroprotective potential of ethanolic extracts of *Z. jujuba* through GC-MS profiling of active components to support the development of new treatments for Parkinson's disease.

The objectives of this study are as follows:

- Characterization of extract components to identify bioactive ingredients through GC-MS
- To investigate the effects of *Z. jujuba* fruit extract on motor behavior performance on rotenone-induced mouse model of neurodegenerative disease
- To analyze its impact on anti-oxidative biomarkers through gene expression profiling.
- To unveil neuroprotective effect of *Z. jujuba* extract on mid-brain Substantia Nigra neurons through histopathological analysis.

CHAPTER NUMBER 2

Literature Review

Parkinson's disease is a neuroprogressive illness which majorly affects older adults. It is the second most common neurological condition after Alzheimer's disease, affecting an estimated 10 million people globally [1]. Motor dysfunctions like bradykinesia, resting tremors, muscle stiffness, and postural imbalance are the major symptoms of PD. Non-motor symptoms like depression, autonomic dysfunctions, sleep disturbance, and cognitive dysfunctioning are also present [6]. Even though the disease progresses gradually, it always leads to significant impairment and reduced the quality of life.

The underlying pathophysiology of Pd is the progressive death of dopamine producing neurons in the SNpc of the midbrain. This cell death results in a major declining of dopamine levels in the striatum, an important area of the brain controlling the motor functions. Low dopamine levels interfere with the basal ganglia-thalamo-cortical circuitry, which is essential for initiating and regulating voluntary movements [57]. Dauer & Przedborski (2003) showed that more than 60–70% of dopamine producing neurons in the SNpc dies off by the time motor symptoms appear clinically, and that dopamine levels in the striatum drop by 80–90% as well. Another significant pathogenic feature is the lewy bodies, which are intracellular clusters of accumulated α -synuclein protein. These accumulations of protein interact with cellular functions and cause neuronal death through mechanisms like oxidative stress, dysfunctioning of mitochondria, and defective protein degradation pathways [58].

Parkinson's disease primarily affects older people, and age is the main risking element for its onset and progression. PD is more common as people age, especially after the age of 60. Its prevalence is estimated to be around 1% in people over 60 and up to 4% in people over 80. PD is associated with a number of neurobiological changes that occur as people age, making the brain more susceptible to neurodegeneration [2]. Although it happens much more slowly than in Parkinson's disease, normal aging is associated with a slow deterioration of dopaminergic

neurons in the substantia nigra. According to research, beginning at age 45, 5–10% of dopaminergic neurons may be lost every ten years [59].

Decreased energy metabolism in neurons, oxidative stress, and damage to mitochondrial DNA are all consequences of aging. One well-known contributing factor to PD is dysfunctioning of mitochondria, which becomes more important as people age. These age-related impairments in mitochondrial function may increase the chances of neurons to PD-related degeneration [60].

Parkinson's disease is a complex neuroprogressive illness which is influenced by both environmental and genetic factors. Mostly the PD cases are classified as idiopathic, meaning that environmental factors and genetic vulnerability interact to cause the disease, even though only about 10–15% of cases are linked to detectable genetic mutations. Exposure to specific environmental toxins, especially pesticides, heavy metals, solvents, and air pollutants, has been shown in numerous epidemiological and experimental studies to significantly increase the risk of PD [6].

The well-known neurotoxic MPTP (1-Methyl-4-phenylpyridinium) and the common pesticide paraquat have structural similarities. Studies on animals have shown that paraquat specifically damages dopamine producing neurons in the SNpc, imitating the pathophysiology of PD. Paraquat induces oxidative stress by producing ROS, which results to mitochondrial dysfunction and neuronal death [61]. Epidemiological evidence indicates a strong correlation between paraquat exposure and PD. A study from the Agricultural Health research cohort found that individuals exposed to paraquat had a significantly higher incidence of PD [62]. Paraquat and the fungicide manab frequently work together. It has been shown that combined exposure significantly increases dopaminergic neuron death, suggesting a cumulative or interaction effect [63].

Rotenone, a naturally occurring insecticide, is a potent inhibitor of complex I, an essential enzyme in the ETC pathway. Because rotenone causes selective declining of dopamine producing neurons and the formation of inclusions that resemble Lewy bodies in mice exposed to it for a long period of time, it is frequently used as a model for Parkinson's disease in research. Rotenone kills dopaminergic neurons by causing oxidative stress and mitochondrial dysfunction. Rotenone primarily damages neurons by blocking complex I of the ETC.

This inhibition disrupts mitochondrial oxidative phosphorylation, reducing ATP synthesis and raising ROS production, resulting to oxidative stress and mitochondrial dysfunctioning. The SNpc's dopaminergic neurons' increased vulnerability to this oxidative damage explains their particular sensitivity in rotenone exposure models. Important pathological and clinical features of PD, including gradual loss of dopamine producing neurons, decreased striatal dopamine levels, motor deficits, and the formation of Lewy body-like inclusions with accumulated α -synuclein, are replicated in rodent models by chronic systemic rotenone administration. Furthermore, rotenone exposure stimulates microglia, which results in neuroinflammation and the release of pro-inflammatory cytokines, exacerbating neurodegeneration [8].

Epidemiological studies have further supported the link between rotenone exposure and higher chances of PD in humans. Environmental toxins play a major role in the progression of sporadic PD, as evidenced by the correlation between occupational exposure to rotenone and related pesticides and higher incidence rates of the disease [62]. Even though the major number of PD cases is sporadic, 5–10% is familial which indicates that genetic factors are a significant contributor to the illness's genesis. The identification of multiple genes connected to both hereditary and sporadic forms of PD by advances in molecular genetics and genome-wide association studies has underscored the importance of genetics in the pathogenesis of the disease [6].

Mutations in those genes that have been conclusively linked to familial PD result in autosomal dominant or recessive inheritance patterns [64]. The SNCA gene encodes α -synuclein, a presynaptic neuronal protein crucial to the pathophysiology of PD. Missense mutations (such as A53T, A30P, and E46K) and gene multiplications that cause dangerous gain-of-function promotes α -synuclein aggregation and Lewy body formation. Leucine-rich repeat kinase 2 (LRRK2) mutations, namely G2019S, are the most frequent cause of autosomal dominant PD, while they can also happen in isolated instances. LRRK2 encodes a kinase involved in autophagy, vesicle trafficking, and cytoskeletal dynamics. Mutations that increase kinase activity cause neuronal toxicity [65].

Autosomal recessive juvenile progression of PD is caused by mutations in the protein. Protein mutations cause autosomal recessive juvenile-onset PD. Parkin is a

proteasomal degradation and mitophagy-related E3 ubiquitin ligase. Loss-of-function mutations, which impact mitochondrial quality control, result in oxidative stress and the accumulation of damaged mitochondria [66, 67].

Mutations in PINK1 (PTEN-induced kinase 1) also cause autosomal recessive PD; PINK1 encodes a mitochondrial kinase that works with Parkin to control mitophagy. PINK1 defects cause neuronal death and disrupt mitochondrial homeostasis [68].

Recessive rare DJ-1 mutations are linked to PD. Protein Deglycase DJ-1 functions as a redox-sensitive chaperone and antioxidant to shield neurons from oxidative stress [69]. Even though most cases of Parkinson's disease are idiopathic, GWAS has identified numerous susceptibility loci that marginally increase the risk of the condition. Examples include: A mutation in the lysosomal enzyme gene GBA (glucocerebrosidase) is the common genetic key factor for PD. These mutations affect lysosomal function and increase α -synuclein aggregation [70]. Variations in the microtubule-associated protein tau, may affect cytoskeletal stability and neurodegeneration, which is linked to the risk of PD [71]. The numerous other risk loci, which include genes linked to immunological modulation, mitochondrial activity, and lysosomal function, highlight PD as a complex disorder influenced by a mix of genetic and environmental factors [72].

Genetic findings have led to improved understanding of PD heterogeneity, improved diagnostic accuracy, and the development of targeted medications. For instance, LRRK2 kinase inhibitors are being studied clinically, and gene therapies or enzyme replacement are being explored for carriers with GBA mutations [73]. By placing electrodes in the subthalamic nucleus or globus pallidus interna, DBS alters neural circuits. It is used to treat patients with motor issues that medicine is unable to adequately manage [74]. Even though they alleviate symptoms, none of the current treatments can halt or reverse the progression of the illness. Long-term use of dopaminergic medications often results in neuropsychiatric side effects, motor fluctuations, and dyskinesias. Additionally, existing medications do not adequately address non-motor symptoms, highlighting the need for novel neuroprotective agents and disease-modifying therapies [40].

Rotenone-induced animal models are commonly used in experimental research to mimic the progressive neurodegeneration observed in PD. In this paradigm, lab animals typically rats or mice are given rotenone, a naturally occurring inhibitor of

mitochondrial complex I. The model is highly flexible for assessing neuroprotective treatments over a variety of time periods since the duration, dose, and route of rotenone exposure may have a major impact on the progression and severity of Parkinsonian symptoms [75, 76].

Rotenone crosses the BBB and selectively targets the nigrostriatal pathway by blocking mitochondrial complex I. This results in the accumulation of ROS, oxidative stress, inflammation, and dopaminergic neuronal death in the SNpc. The resulting striatal dopamine depletion causes motor impairments such as bradykinesia, stiffness, and tremors, which are comparable to those seen in human PD [77].

The effects of rotenone are typically examined in animal experiments over a range of time periods, from a few days to several weeks, depending on the study's objectives: Brief exposure to rotenone is commonly used to study early cellular and molecular processes like mitochondrial failure, ROS production, and the first signs of neuroinflammation. Changes in behavior may not be apparent at this time. For example, early markers of oxidative stress and inflammatory cytokines can be evaluated three to five days following therapy [78]. During this time, animals begin to show early motor deficits and dopaminergic neuron loss. This is a common window for evaluating potential neuroprotective medications because treatments may be used to see if they can reverse the course of the disease. For example, resveratrol and quercetin have shown protective benefits against dopaminergic neurotransmitters when taken with rotenone for two to three weeks [79].

Extended use results in progressive neurodegeneration, Lewy body-like inclusions (α -synuclein aggregation), and severe motor and cognitive impairments. This model length is used for long-term clinical studies because it best captures the entire pathogenic range of Parkinson's disease. For example, long-term co-treatment with compounds like *Mucuna pruriens* or coenzyme Q10 has shown long-lasting protective benefits in 28-day models [80, 81]. In a novel method, they continuously injected rotenone (3 mg/kg/day) into Lewis rats for 33 days using subcutaneously implanted Alzet osmotic mini-pumps. This approach produced Lewy body-like inclusions and selective loss of dopamine producing neurons in the nigrostriatal pathway, which closely mirrored the pathophysiology of PD in humans [82].

Rotenone (3 mg/kg/day) was administered intraperitoneally to Wistar rats. These rats' behavioral abnormalities and the substantial neuronal death in the SNpc over time demonstrated that PD-like symptoms had been successfully induced in them [83]. By injecting rotenone directly into specific brain regions, such as the SNpc, it has been used to induce localized dopaminergic neuron degeneration. This method can be used to investigate region-specific neurodegeneration and associated behavioral changes [84]. Using subcutaneously implanted osmotic mini-pumps, C57BL/6J mice were exposed to low-dose rotenone (2.5 mg/kg/day) for four weeks, resulting in dopaminergic neurodegeneration, including α -synuclein accumulation in the SNpc, motor deficits, and gastrointestinal problems [85].

Ziziphus jujuba Mill a Rhamnaceae family member, is commonly used in ancient medicine, particularly in Asia, and is also known as jujube or red date. The neuroprotective potential of *Z. jujuba*, a plant known for its diverse range of pharmacological efficacies, has attracted a lot of attention because of its rich phytochemical composition, which includes polysaccharides, alkaloids, flavonoids, and saponins. The neuroprotective properties of this plant have been investigated in relation to a variety of neurological conditions, such as epilepsy, ischemic stroke, PD, and AD [53].

A number of *Z. jujuba* components offer neuroprotection in different ways. Triterpenic acids, polysaccharides, vitamin C, and flavonoids (like quercetin and kaempferol) are all abundant in *Z. jujuba* fruit. These compounds contain antioxidant, anti-inflammatory, and anti-apoptotic qualities [86]. Spinosin and jujubosides A and B are found in *Z. jujuba* seeds. Through the regulation of the GABAergic and serotonergic systems, these are recognized for their calming, anxiolytic, and memory-enhancing actions [87]. Alkaloids and phenolic acids found in leaves and bark have antioxidant properties and lower neuroinflammation [88].

The hallmarks of AD include β -amyloid plaque, oxidative stress, tau hyperphosphorylation, and cholinergic dysfunction. Because the polyphenols in *Z. jujuba* fruit enhance natural antioxidant enzymes like SOD and catalase and scavenge free radicals, the fruit extracts show less oxidative stress [89]. In models of scopolamine-induced cognitive impairment, spinosin, a flavonoid glycoside extracted from the seed, significantly improves memory by increasing acetylcholine levels and changing the cholinergic system [90]. In brain tissues, jujube

polysaccharides reduce pro-inflammatory cytokines (TNF- α , IL-6) [91]. The anticonvulsant properties of *Z. jujuba* seeds are linked to GABA-A receptor modulation. In models produced by pentylenetetrazol (PTZ), jujuboside A inhibits seizure activity and raises GABA levels [87]. Spinosin shields hippocampal neurons from kainic acid-induced excitotoxicity [86]. *Z. jujuba* seed extracts improve neurological scores, reduce neuronal death, and lessen the degree of cerebral infarction in models of ischemia-reperfusion damage. It boosts brain-derived neurotrophic factor production, which encourages neurogenesis and post-injury synaptic plasticity.

Parkinson's disease is mostly related to oxidative imbalance, destruction of mitochondria, and the gradual loss of dopaminergic neurons. Conventional medications can effectively treat symptoms, but they cannot save neurons or change the progression of the disease, which has led to research into natural remedies. Because they produce a wide variety of secondary metabolites, medicinal plants have developed into captivating choices in this regard. *Zizyphus jujuba*, a fruit long valued for its medicinal properties, contains bioactive components such as flavonoids, saponins, and alkaloids that are thought to have antioxidant and neuroprotective qualities. Its efficacy in Parkinsonian models, however, is currently not well supported by experiments. The present study examined the neuroprotective effect of *Z. jujuba* ethanolic extract against rotenone-induced Parkinsonism in mice by combining behavioral, biochemical, histological, and GC-MS techniques.

CHAPTER NUMBER 3

Methodology

The purpose of the study was to guarantee that data pertinent to the research topic would be accurately collected, analyzed, and interpreted. Various [qualitative/quantitative/mixed-method] techniques were employed, contingent on the study's particular objectives and the type of data. The adequacy of each approach for answering the study questions and guaranteeing the validity and reliability of the findings guided its selection. Details on the materials utilized, data collection methods, statistical analysis, and experimental design are all included in the protocols outlined below.

3.1: Collection and Identification of Plant Material

Ziziphus jujuba was collected from reliable sources. It was collected at the right time of year to increase the amount of phytochemicals. By ensuring the integrity of study, a certified botanist carried out the appropriate authentication and documentation of plant species.

3.1.1: Preparation of *Zizyphus jujuba* Ethanolic Extract

The ethanolic extract was prepared by following the protocol involving the collection of *Z. jujuba* fruits. The fruit part was dried in an incubator and then it was converted to powdered form. For extract preparation 10g of plant powder was treated with 70% ethanol. For this purpose 70ML ethanol and 30ML autoclaved distilled water was added to plant powder in flask. Then it was shifted to orbital shaker at 37 degree Celsius temperature and 200rpm for 24 hours. After removing from shaker the material was filtered through whatman No 1 filter paper. This material was shifted to beaker that was covered with filter paper having pores on it. After that it was shifted to vacuum oven for drying purpose at 40 degree Celsius temperature for almost 1 week. After that the sticky material was extracted and shifted to empty falcons with measured weight. Then this extract was stored at 4 degree Celsius for further use [92].

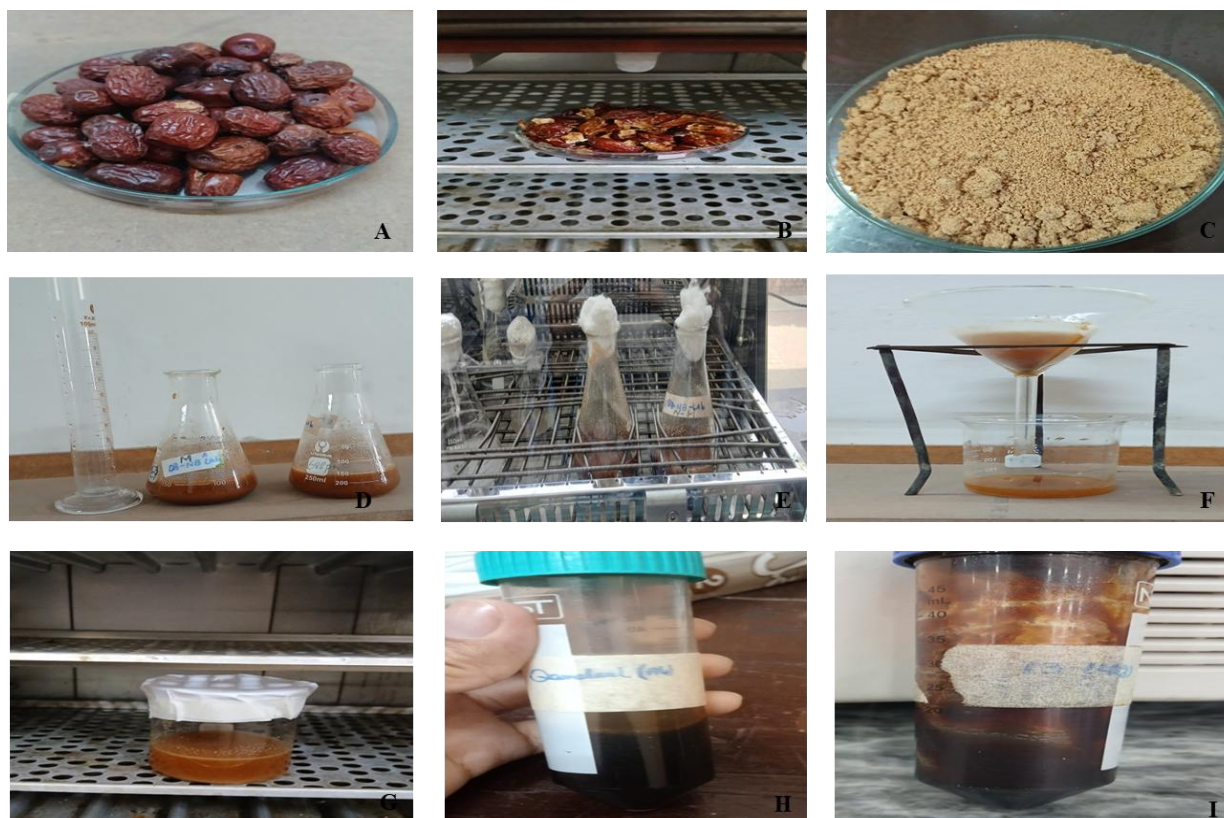


Figure 3.1.1: Extract Preparation. (A): *Ziziphus jujuba* Fruit. (B): Removal of Seeds and drying in an incubator. (C): Powder of Fruit. (D): Ethanol mixing. (E): Orbital Shaker. (F): Filtration. (G): Incubator drying. (H): Sticky liquid. (I): Lyophilized extract.

3.1.2: Extract Profiling

The bioactive ingredients of the ethanolic extract of *Ziziphus jujuba* fruit were investigated using Gas Chromatography–Mass Spectrometry. The evaluation was conducted using an Agilent 7890B gas chromatograph and an Agilent 5977A mass selective detector fitted with an HP-5MS fused silica capillary column (30 m × 0.25 mm internal diameter, 0.25 µm films thick, 5% phenyl methylpolysiloxane). A 1 µL aliquot of the ethanolic extract was injected in split mode with a split ratio of 10:1, while helium was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector's temperature was set at 250 °C. The oven temperature program ramped up to 200 °C at a rate of 10 °C per minute after beginning at 60 °C for two minutes. It then stayed there for five minutes. After that, it was increase by 5 °C per minute to 280 °C and stayed there for 15 minutes. The mass spectrometer was operated in

electron impact (EI) ionization mode at 70 eV, with an ion source temperature of 230 °C, quadrupole temperature of 150 °C, interface temperature of 280 °C, and a scan range of m/z 40–600. Following the automatic integration of chromatographic peaks, the substances were identified by comparing their mass spectra with those in the NIST/EPA/NIH Mass Spectral Library (NIST version 14/20) and with published literature. Compounds that scored 90% or higher on the similarity scale were considered reliable. The peak area normalization technique was used to check the relative abundance of each detected constituent without the use of correction factors. The results were then presented as the percentage composition of the extract [93].

3.2: Animal Use

Male Swiss albino mice (*Mus musculus*) aged 6–8 weeks and weighing 25–30 grams were used in the study. All animals were acquired from a registered laboratory animal breeding facility and acclimated to laboratory conditions for at least one week before the experimental procedures started. Mice were deemed healthy, active, and free of any visible anomalies or signs of disease prior to their inclusion in the study.

The animals were kept in polypropylene cages with sterile bedding in standard conditions, which included a 12-hour light/dark cycle, a temperature of $22 \pm 2^\circ\text{C}$, and a relative humidity of $55 \pm 10\%$. They are fed with standard pellet diet and have unlimited access to filtered tap water. Every two to three days, cages were cleaned and bedding was replaced to maintain hygienic conditions and lessen stress (Fig: 3.2). Throughout the trial, every effort was made to ensure the animals' comfort. Mice were handled carefully to reduce stress, and they were frequently watched for any signs of illness or odd behavior. Animals displaying signs of severe distress or suffering were to be humanely put down, even though no such incidents occurred during the trial.



Figure 3.2: Male Swiss Albino Mice

3.3: Grouping and dosing

Male Swiss Albino mice were randomly collected and allocated to the following groups.

Group 1: Vehicle Control (n=6) (1ml/kg of DMSO in sunflower oil)

Group 2: Rotenone treated group (2.5mg/kg in DMSO containing sunflower oil)

Group 3: 2.5mg/kg rotenone + *Eth Z. jujuba* (200mg/kg)

Group 4: 2.5mg/kg rotenone + *Eth Z. jujuba* (400mg/kg)

Group 5: *Eth Z. jujuba* 400mg/kg + Vehicle (DMSO in sunflower oil)

Group 6: 2.5mg/kg rotenone+ Sinemet 20mg/kg

3.4: Mice dose administration

Rotenone was dissolved in a mixture of sunflower oil and dimethyl sulfoxide (DMSO). A common solvent ratio that ensures total solubility and reduces injection site discomfort is DMSO: Sunflower oil = 1:9 or 1:4. The final concentration was adjusted to provide the required dosage per body weight. The solution was prepared in poorly lit vials and stored in amber containers to prevent deterioration. Rotenone was injected subcutaneously into the interscapular region once daily (Fig: 3.3 A).

The literature states that the dosage was 1.5–2.5 mg/kg body weight per day for 21 consecutive days. The injection volume was restricted to 0.1 mL per 10 g body weight in order to prevent pain. Mice were monitored for signs of discomfort or reaction after injection. Because rotenone is dangerous, it must be handled in a ventilated area with PPE (gloves, mask, and lab coat) [94].

An oral ethanolic extract of *Zizyphus jujuba* was administered to evaluate its neuroprotective potential against rotenone-induced neurotoxicity. A specific quantity of the dried extract was reconstituted in autoclaved distilled water to ensure a constant suspension. The suspension was fully vortexed and made fresh daily for the best dispersion. The dose concentration was calculated based on the animal's body weight.

The extract was gavaged into the mouth using a soft oral feeding needle (Fig: 3.3 B). The dosage ranged from 200 to 400 mg/kg body weight per day, depending on the experimental group and the information at hand. Administration was carried out once daily for the same duration as rotenone exposure (e.g., 21 days). The gavage volume was kept below 10 mL/kg body weight to avoid gastric overload, which is typically ~0.2 mL/mouse. Every medication was administered at the same time each day, preferably in the morning, to reduce circadian variance. Mice were weighed twice a week in order to adjust the dosage volume. Clinical observations for abnormal behaviors, tremors, or weight loss were recorded daily [95].

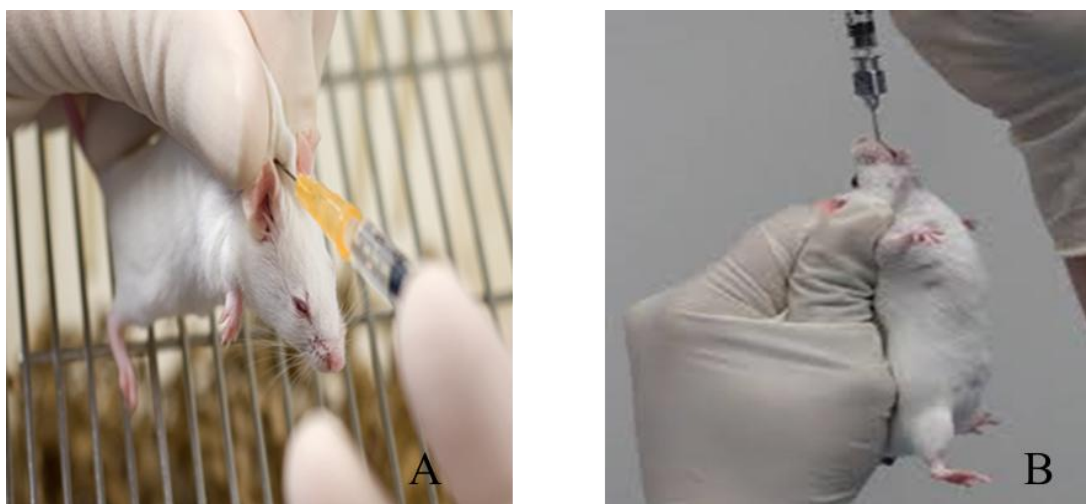


Figure 3.3: Dosing Method: **(A):** Subcutaneous dosing. **(B):** Oral dosing.

3.5: Behavioral Assessment

A crucial method for determining functional impairments and the progression of neurodegeneration in animal models of PD is behavioral evaluation. A group of behavioral tests were used in this study to fully observe the motor and non-motor activity domains that are frequently impaired in Parkinsonian disease. These tests were selected to evaluate key behavioral traits, such as locomotor activity, coordination, muscular stiffness, bradykinesia, postural stability, and abnormalities in gait, that are closely similar to the symptoms of PD in humans.

The integration of multiple tests enables multidimensional analysis. To detect hypoactivity and anxiety-related changes, for instance, which are commonly connected to dopaminergic neuronal loss in the nigrostriatal circuit, general locomotion and exploratory behavior were observed. Tests such as the pole test and climb-down test provide sensitive indicators of bradykinesia and axial motor deficiencies by evaluating postural reflexes and coordinated movements. In gait analysis, stride length measurement is very useful in detecting mild impairments in motor control and stride regularity, which are traits of Parkinsonian gait.

Together, these behavioral techniques offer insights into the severity and progression of motor dysfunctions as well as functional correlates of biochemical and histological changes in the brain. Importantly, behavioral outcomes are necessary to evaluate the efficacy of neuroprotective agents, such as the *Zizyphus jujuba* extract utilized in this study. Improvements or the preservation of typical behavior after treatment are indicative of potential therapeutic benefits and are often supported by associated molecular and neurochemical findings. The goal of the project is to use this multimodal behavioral approach to show a reliable relationship between neurodegeneration and observable behavioral changes, thereby offering a translational bridge to human clinical symptoms of PD.

3.5.1: Open Field Test

In mice models of neurodegenerative diseases like Parkinson's disease, the Open Field Test (OFT) is frequently used to monitor the exploratory behavior, locomotor activity, and anxiety-related reactions. In order to reduce visual distractions and hinder escape, the apparatus usually consists of a square or circular arena with opaque walls. The standard dimensions are 50 × 50 × 25 cm. A white sheet with 16 equal squares was placed [96-97] (Fig: 3.5.1). To prevent behavioral changes

brought on by too much brightness, moderate lighting (20–60 lux) is utilized. Animals are usually accustomed to the surroundings for at least half an hour before testing, and the area is cleaned with 70% ethanol in between sessions to eliminate smell cues.

During the test, each mouse is placed in the middle of arena, marked with black color on its neck and allowed to explore freely, usually for 5 minutes [98]. In this experiment a camera captured a activities of each mouse for 5-minutes. ToxTrac tracking software was used to examine locomotor patterns. The number of each box entry was measured by manually.



Figure 3.5.1: Open Field Box

3.5.2: Narrow Beam Walk Test

The Narrow Beam Walk Test is a sensitive behavioral test which is used to monitor balance, motor coordination, and fine motor function in mouse models of neuroprogressive disorder like PD. The device typically consists of a 1-meter-long beam elevated 50–60 cm above a cushioned platform to prevent injuries in the event of a fall (Fig: 3.5.2). To provide increasing challenge and encourage movement toward a goal area, the beam, which is often illuminated by a 40–60 W light source, can be uniformly wide or composed of gradually narrowing segments, usually ranging in size from 3.5 to 0.5 cm [99]. Pre-training usually lasts two to five days, during which the animals are gradually exposed to narrower beams and shorter initial distances from the target area. Young mice typically reach performance

stability in three days, but older or neurologically impaired mice may take up to five days [100].

During testing, a mouse is kept at the lit beginning end of the beam and instructed to move toward a goal box or home cage that is enclosed and darker. To lessen external influences, the goal area and beam are cleaned with 70% ethanol in between trials. Additionally, there are documented cases of immobility, including hesitations or pauses [100]. In addition to recording immobility periods (the times the animal pauses or hesitates), trials are often stopped at 60 seconds if the animal fails to meet the goal. For a more thorough evaluation, some methods employ thin mesh overlays to detect limb slippage through the grids.

The beam walk test is well-respected in preclinical neurobehavioral research because it can detect mild motor coordination impairments that other tests, such as the rotarod or open field tests could overlook [101].

For this investigation, two separate trials were recorded for each mouse using a digital camera setup. After that, the video was analyzed, and average values were calculated to ensure precision and consistency in the assessment of motor performance.



Figure 3.5.2: Narrow Beamwalk Apparatus

3.5.3: Poleclimb down Test

A vertical pole that is typically 50 cm in height and 1 cm in diameter is used in the Pole Climb-Down Test, which is frequently used to assess bradykinesia, motor control, and coordination in rat models of neurological disorders (Fig: 3.5.3). The test involves placing mice facing up at the top of the pole and instructing them to

turn and fall head first into a cushioned platform. Important performance indicators include the number of slips or falls, the total time spent descending (T_{descend} or T_{total}), and the time it takes to begin the turn (T_{turn}). The term "maximum impairment" refers to falling or failing to descend within a set amount of time, typically 60 or 120 seconds.

Animals undergo two to five training sessions over one or two days to ensure task familiarity and minimize anxiety-related variability. The latencies and any slip or fall incidents are averaged for statistical reliability. When an animal stops during a trial, it is rejected and the experiment is repeated. The maximum cutoff time (e.g., 120 s) is set for abrupt drops.

Longer descent times, different turn latencies, and higher error rates are some of the motor deficits that this test can identify as being indicative of neurodegenerative models, such as Parkinsonism and stroke brought on by MPTP or rotenone. Neuroprotective or dopaminergic treatments can help to partially restore these deficits [102].



Figure. 3.5.3: Pole Climbdown Apparatus

3.5.4: Tail Suspension Test

The Tail Suspension Test (TST) is a frequently used behavioral test to evaluate depressive behavior and the efficacy of antidepressant drugs in rodents, primarily mice. The TST, which was first introduced by Steru et al. (1), is conceptually similar to the Forced Swim Test (FST) and is founded on the behavioral despair principle. During this test, animals are lifted by their tails, and their movements are recorded. The length of immobility serves as a proxy for depressive-like states in animal models, serving as a sign of behavioral inhibition or hopelessness.

Each mouse is held separately during the typical TST procedure using adhesive tape that is affixed approximately 1 to 2 cm from the tip of the tail. Because the tape is attached to a horizontal bar or shelf, the mouse can hang freely at a preset height (typically about 50 cm) above the floor or a cushioned surface (Fig: 3.5.4). The entire test usually lasts six minutes, during which the animal's behavior is either observed in person or recorded on video.

The primary behavior that is evaluated is the duration of immobility, which is the absence of initiated motions (apart from passive swinging due to momentum). Periods of struggle or escape-oriented activity are viewed as active, whereas complete immobility is indicated by a complete stop in movement. Antidepressant treatments or substances with potential antidepressant-like effects typically significantly reduce immobility time, indicating increased desire or escape-directed behavior [103-104].



Figure. 3.5.4: Tail Suspension Apparatus

3.5.5: Stride Length Test

In mouse models of neuroprogressive disorder like PD, Huntington's disease, and cerebellar ataxia, locomotor function, motor coordination, and gait impairments are commonly evaluated using a behavioral test known as the Stride Length Test. A quantitative evaluation of motor impairments is provided by measuring the length of each step an animal takes when walking. This non-invasive, user-friendly test is sensitive to mild motor impairments, especially in forelimb and hindlimb coordination.

The test involves placing the mouse on a runway or narrow hallway, which is typically 50–100 cm long and 5–10 cm wide, and allowing it to move freely between the two ends (Fig: 3.5.5). The animal's hindpaws are often dipped in non-toxic, water-soluble ink of different colors (mostly red before the experiment) to enable the clear viewing and differentiation of paw prints on a piece of white paper laid out along the hallway floor. As the animal crosses the runway, a trail of its footsteps is left behind. These footprints are then manually inspected. The mean stride length is usually calculated by evaluating three to five complete strides per limb. Particularly in Parkinsonian models, shorter strides are interpreted as an indication of bradykinesia or motor dysfunction [105-106].



Figure 3.5.5: Stride Length Test Apparatus

3.5.6: Stepping Test

The stepping test is a widely used behavioral test to evaluate bradykinesia and akinesia in rodent models of PD, particularly in rats. Animals with the degeneration of dopamine producing neurons specifically in the nigrostriatal pathway move their forelimbs less effectively and take fewer adjustment steps when traveling laterally, which is the basis for this test. In the stepping test, the animal is gently held so that only one forelimb touches the ground and the other is restrained while being moved sideways over a predetermined distance (Fig: 3.5.6). The number of adjustment steps of the unconstrained forelimb is counted. A marked reduction in the number of steps is a sign of impaired motor function, which is a hallmark of PD.

Its sensitivity is highlighted in detecting forelimb akinesia and asymmetrical motor impairments following unilateral 6-hydroxydopamine (6-OHDA) lesions [107]. Moreover this test was used to assess behavioral changes in rats with dopaminergic lesions and found that dopaminergic medications improved stepping performance, validating the test's utility in assessing the progression of PD and the efficacy of therapy. Furthermore, dopamine loss in the striatum has been linked to the stepping test, indicating a robust neurochemical foundation for reported motor deficits [108]. Moreover, the stepping test has been associated with dopamine depletion in the striatum, suggesting a strong neurochemical basis for documented motor impairments. Because it is non-invasive and sensitive to minute motor changes, this test is not only repeatable but also appropriate for long-term research.

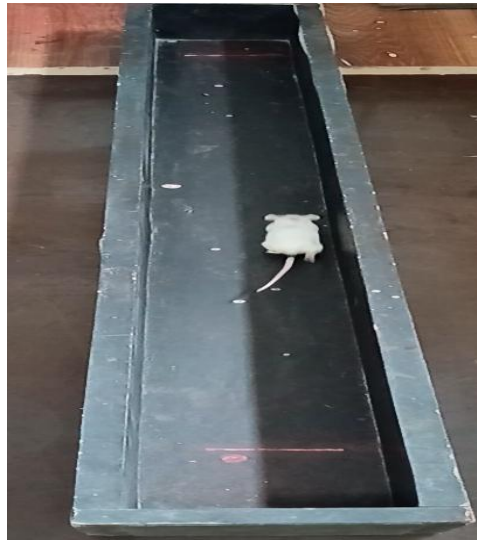


Figure 3.5.6: Stepping Test Apparatus

3.6: Histological Examination

After experimental procedures (like behavioral testing or medication delivery) are completed, mice are killed in compliance with ethical standards. The brain is quickly removed and prepared for histological analysis in order to assess neurodegeneration, particularly in the substantia nigra, a critical area affected by PD.



Figure 3.6: Mice surgery: (A): Dead Mice. (B): Mice brain.

3.6.1: Fixation and Perfusion

After the mice have been thoroughly sedated with a suitable anesthetic (such as ketamine/xylazine), transcardial perfusion is performed. The brain tissue is fixed with 4% paraformaldehyde (PFA) in PBS after the blood has been extracted using

cold phosphate-buffered saline (PBS, pH 7.4). After perfusion, the entire brain is carefully removed and post-fixed in 4% PFA for 24 to 48 hours at 4°C [109].

3.6.2: Cryoprotection

The brain is cryoprotected in 30% sucrose in PBS until it sinks (24–48 hours) after fixation to prevent freezing artifacts during sectioning [110].

3.6.3: Sectioning

The brain is embedded in a compound with the ideal cutting temperature. Using a cryostat or freezing microtome, coronal sections 10–20 µm thick are cut, especially from the midbrain region (~bregma -2.8 mm to -3.8 mm), which contains the substantia nigra pars compacta (SNpc). Sections are put on poly-L-lysine-coated glass slides and allowed to air dry [111].

3.6.4 Procedure for Hematoxylin and Eosin (H&E) Staining.

The following procedure is used to dye the mounted brain sections:

3.6.4.1: Staining:

Spend five to ten minutes submerged in hematoxylin solution. Rinse for five minutes under running water. To distinguish, dip in acid alcohol (1% HCl in 70% ethanol) for a short while. Rinse once more under running water to finish the blueing process (either Scott's tap water alternative or 0.2% ammonia water). Eosin Y counterstain for one to two minutes. Rinse with distilled water for a short time [112, 113].

3.6.4.2: Mounting and Dehydration:

One minute for 70% ethanol, one minute for 95% ethanol, and two minutes for 100% ethanol Xylene I and II: two by three minutes Cover with a coverslip after mounting with DPX or any other appropriate mounting media.

3.6.4.3: Microscopic Examination

Utilize a light microscope to examine the stained sections. The ventral midbrain has a pigmented area known as the substantia nigra. Large, spherical nuclei and tightly packed neurons make up the SNpc in control sections. In Parkinsonian models (such as those treated with rotenone or 6-OHDA), the SNpc exhibits gliosis, vacuolation, cell shrinkage, and neuronal death. ImageJ or other morphometric analysis tools can be used to quantify neuronal density or degeneration [114].

3.7: Quantitative Real-Time PCR (qPCR) Analysis

3.7.1: RNA Extraction

Total RNA extraction is a crucial step in molecular biology research because it provides the template for later procedures like reverse transcription and gene expression analysis. The widely used method is TRIzol, or phenol-chloroform extraction. To put it briefly, 1 mL of TRIzol reagent is used to collect and homogenize samples of brain tissue in 50–100 mg of tissue on ice. The homogenate is incubated for five minutes at RT to enable the complete breakdown of nucleoprotein complexes.

After adding 0.2 mL of chloroform for every 1 mL of TRIzol, the sample is shaken violently for 15 seconds and incubated at RT for two to three minutes. By centrifuging at $12,000 \times g$ for 15 minutes at 4°C, phase separation is accomplished, producing an organic phase with proteins, an interphase with DNA, and an aqueous phase with RNA. After carefully transferring the upper aqueous phase to a new tube free of RNase, 0.5 mL isopropanol per 1 mL TRIzol is used to precipitate the RNA. The RNA pellet is centrifuged ($12,000 \times g$, 10 min, 4°C), rinsed with 75% ethanol, allowed to air dry for a short time, and then dissolved in water devoid of RNase. Agarose gel electrophoresis is used to validate the quality and purity of RNA, which are evaluated spectrophotometrically at the A260/A280 ratio [115].

3.7.2: cDNA Synthesis

Reverse transcriptase enzymes are used in complementary DNA (cDNA) synthesis to create stable DNA templates from extracted RNA. Reverse transcription requires high-quality RNA (1–2 µg), and the reaction is conducted with either random hexamer primers, which offer broader coverage including non-polyadenylated transcripts, or oligo(dT) primers, which selectively anneal to the poly(A) tails of mRNA. RNA template, 1 µL of oligo (dT) or random primers, 1 µL of dNTP mix (10 mM each), and nuclease-free water make up a standard reaction mixture (20 µL). After five minutes of heating to 65°C, the mixture is cooled on ice. Next, 200 U of reverse transcriptase enzyme (such as M-MLV or SuperScript II/III), reverse transcriptase buffer, and RNase inhibitor (20 U) are added. After 60 minutes of incubation at 42–50°C, the enzyme is inactivated for 10 minutes at 70°C. The produced cDNA can be stored at -20°C for later use or used right away for subsequent processes like PCR and qPCR [116-1118].

3.7.3: qPCR Process

To measure the relative gene expression in cDNA extracted from mouse brain tissue, qPCR was carried out by using a SYBR Green detection system. It contained 5.0 μL of 2 $\times$ SYBR Green Master Mix (Thermo Fisher Scientific, USA), 0.4 μL of forward and reverse primers (10 μM stock; final concentration 400 nM), 2.0 μL of diluted cDNA template (equivalent to roughly 1–10 ng single-stranded cDNA), and nuclease-free water to volume. The reactions were prepared on ice in a total volume of 10 μL .

To reduce pipetting variation, a master mix was made for every target or reference gene. It was then aliquoted into the wells of a 96-well optical qPCR plate, with cDNA added last (Fig: 3.7). After being sealed and briefly centrifuged, the plates were put into an Applied Biosystems StepOnePlus™ real-time PCR thermocycler. The cycling program included a 3-minute initial denaturation at 95 °C, 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 30 s. A melt-curve analysis from 65 to 95 °C was carried out after amplification to verify the formation of a single, distinct product. To identify contamination or genomic DNA carryover, respectively, no-template controls (NTC) and no-reverse-transcriptase (no-RT) controls were added to each primer set.

For every biological sample, all reactions were performed in triplicate (technical replicates). Prior to experimental runs, primer specificity and efficiency were confirmed using standard-curve analysis over a 5-point, 10-fold serial dilution of pooled cDNA. An acceptable efficiency was defined as 90–110% and a correlation coefficient (R^2) ≥ 0.99 . The $2^{-\Delta\Delta\text{Ct}}$ method was used to calculate the levels of gene expression. The mean ΔCt of the control group served as the calibrator, and ΔCt values were normalized to the geometric mean of two validated reference genes. ΔCt values were subjected to statistical analyses; the results are presented as mean $\pm$ SEM [119].

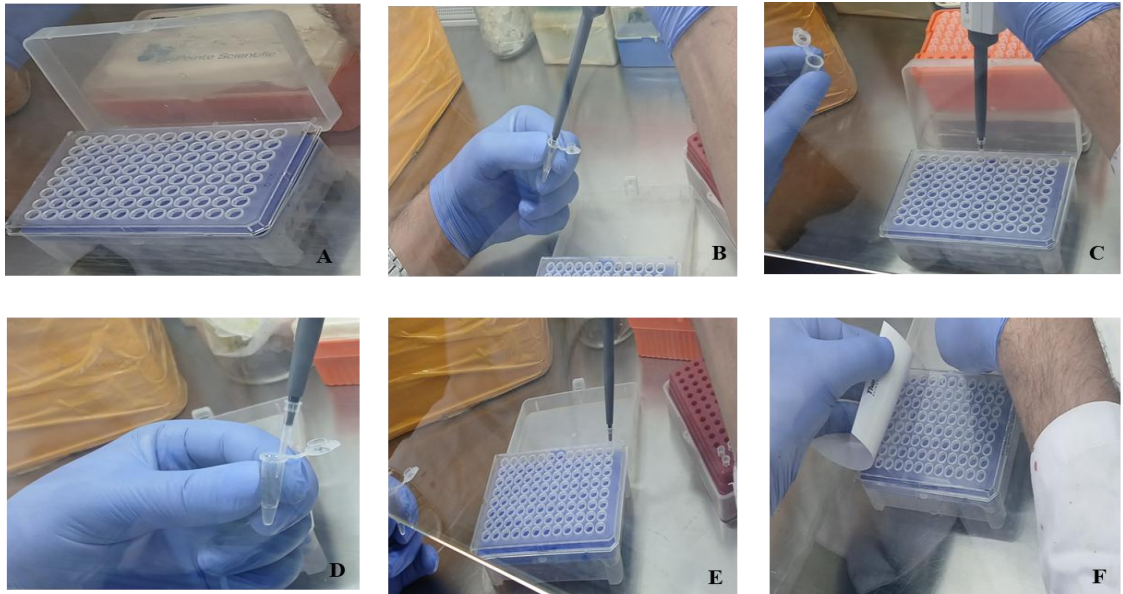


Figure 3.7: qPCR Procedure (A): 96 Wells plate. (B): cDNA dilution. (C): Primers loading. (D): Sample collection. (E): Sample mixing. (F): Plate sealing.

3.8: Statistical Analysis

The mean $\pm$ standard error of the mean (SEM) was used to interpret all the experimental results. To ascertain the degree of significance between individual group means, statistical comparisons among several groups were conducted using one-way analysis of variance (ANOVA) and Bonferroni's post hoc test. This technique was used to account for type I error from multiple comparisons and determine whether there was a statistically significant difference between the experimental groups. GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA) was used to conduct the analysis. Statistical significance was defined as a p-value of less than 0.05.

CHAPTER 4

Results

The main purpose of the current analysis was to determine the phytochemical bioactive compounds present in the ethanolic extract of *Zizyphus jujuba* affects a mouse model of PD brought on by rotenone. First, as a broad measure of health, toxicity, and the advancement of illness, variations in body weight were noted during the trial. An early indicator of the systemic effects of rotenone or the therapeutic potential of the plant extract was a steady drop or improvement in body weight.

Second, a group of several behavioral tests, such as the open field test, pole test, narrow beam walk, tail suspension test, stride length and stepping test were used to measure motor coordination, locomotor activity, and depressive-like behavior. These experiments demonstrated the extract's possible therapeutic benefits as well as the neurological deficits brought on by rotenone.

Thirdly, hematoxylin and eosin stained brain slices were subjected to histological investigation, with a particular focus on the substantia nigra area, in order to identify any protective changes brought about by the therapy, as well as neuronal degeneration and structural damage. The cellular architecture of several groups was seen and compared using hematoxylin staining.

At last the analysis of bioactive stress biomarkers was done by gene expression in SNPc of all treated groups including rotenone induced model and extract treated groups.

4.1: Neuroprotective efficacy of *Ziziphus jujuba*

4.1.1: Ethanolic extract Yield (%) of *Z. jujuba*

The usual range for percentage yield is 10% to 20%. The ethanolic extract of *Z. jujuba* fruit was prepared by following the protocol by mixing the fruit powder in ethanol and drying it in an incubator. After lyophilizing it produced a 15.2% yield.

4.1.2: Phytochemical Profiling of *Ziziphus jujuba* Extract Using GC-MS

Fatty acids, sterols, alcohols, aldehydes, flavonoids, and other aromatic compounds are among the many bioactive phytochemicals that are usually detected by GC-MS analysis of *Ziziphus jujuba* (fruit ethanolic extract).

The brief list of chemical constituents present in *Ziziphus jujuba* is mentioned in the table (4.1).

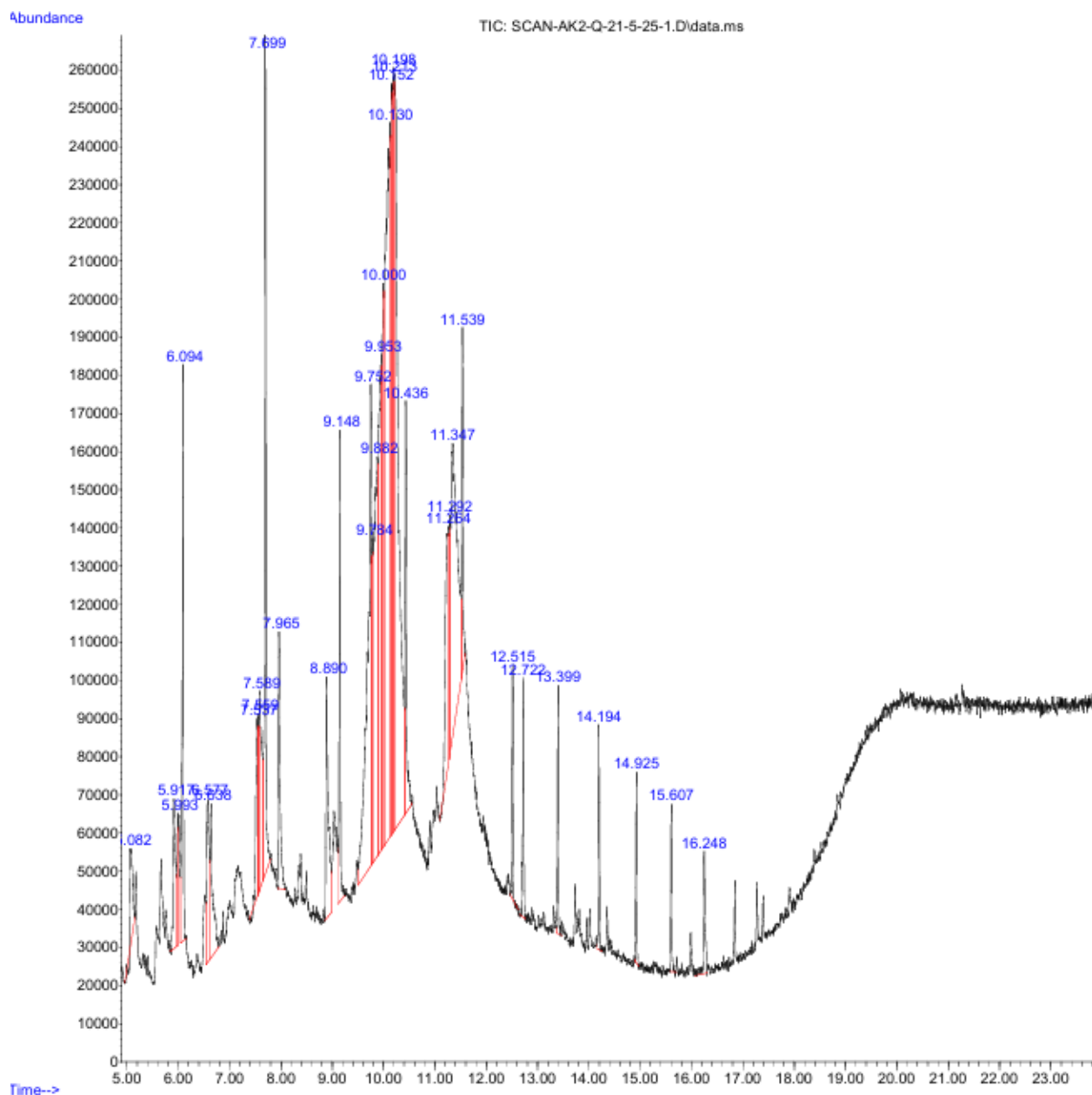


Figure 4.1: Chromatogram of ethanolic extract of *Z. jujuba* fruit.

Table. 4.1: Compounds detected in the ethanolic extract of *Ziziphus jujuba* by GC-MS.

Sr No	RT	Name of Compounds	Molecular formula	MW	Peak Area %
1	5.08 2	2-Hydroxy-gamma-butyrolactone	C ₄ H ₆ O ₃	102.09	0.92
2	5.91 7	1-(2-Thienyl)-1-octanone	C ₁₂ H ₁₈ OS	210.33 6	1.38
3	5.99 3	Cyclopentasiloxane, decamethyl-	C ₁₀ H ₃₀ O ₅ Si ₅	370.77	0.62
4	6.09 4	3,3,4-Trihydroxyphenylalanine, 5TMS	C ₂₅ H ₆₆ N ₄ Si ₅	563.24 7	2.75
5	6.57 7	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	C ₆ H ₈ O ₄	144.12	1.56
6	6.63 8	1H-Indene, 2,3-dimethyl-	C ₁₁ H ₁₂	144.21	1.45
7	7.53 7	5-Hydroxymethylfurfural	C ₆ H ₆ O ₃	126.11	1.04
8	7.55 9	5-Hydroxymethylfurfural	C ₆ H ₆ O ₃	126.11	0.68
9	7.58 9	5-Hydroxymethylfurfural	C ₆ H ₆ O ₃	126.11	2.12
10	7.69 9	2-Chloro-4-(4-methoxyphenyl)-6-(trifluoromethyl)pyrimidine	C ₁₇ H ₁₂ ClF ₃ N ₂ O	352.74	3..97
11	7.96 5	8-Azabicyclo[3.2.1]octane	C ₇ H ₁₃ N	111.18	1.53
12	8.89 0	1-Methylnaphthalene	C ₁₁ H ₁₀	142.2	2.16
13	9.14 8	8-Methoxy-2,3-dihydro-1,4-benzodioxin-5-yl)methanol	C ₈ H ₈ O ₂	196.19 6	1.89
14	9.75 2	2,4-Di-tert-butylphenol	C ₁₄ H ₂₂ O	206.32	6.91

15	9.78 4	Lethane	C9H17NO2S	203.30	1.14
16	9.88 2	tert-Butoxypropan-2-yl 2-methylpropanoate	C12H22O3	206.31	5.52
17	9.95 3	tert-Butoxypropan-2-yl 3-methylbenzoate	C15H23O4	267.33 4	5.44
18	10.0 0	1-(4-Morpholinyl)ethanone	C6H11NO2	129.16	4.22
19	10.1 30	(2R,4S)-2-(Hydroxymethyl)tetrahydrofuran-3-ol	C5H10O3	118.13	13.75
20	10.1 52	1-(4-Morpholinyl)ethanone	C7H11NO2	131.18	3.92
21	10.1 98	D-erythro-Pentose, 2-deoxy-	C5H10O4	134.13	4.62
22	10.2 13	2-Deoxy-D-erythro-pentose	C5H10O4	134.15	12.34
23	10.4 36	2,5-Dihydroxybenzoic acid, 3TMS derivative	C7H6O4	154.12	2.08
24	11.2 64	5-Methyl-2,3-dihydro-1H-indene	C10H12	132.20	3.08
25	11.2 92	Thiophene, tetrahydro-3-methyl-	C5H10S	102.19	1.07
26	11.3 47	Benzene, (1-methylenepropyl)-	C10H12	132.20	6.66
27	11.5 39	Cyclononasiloxane, octadecamethyl-	C18H54O9Si9	667.38 55	1.51
28	12.5 15	Pentasiloxane, dodecamethyl-	C12H36O4Si5	384.84	0.83
29	12.7 22	2,5-Cyclohexadien-1-one, 2,6-bis(1,1-dimethylpropyl)-4-methyl-	C17H30O	250.41	0.97
30	13.3	2,4-	C13H22O3Si2	282.48	0.93

	99	Bis(trimethylsilyloxy)benzoic acid trimethylsilyl ester		6	
31	14.1 94	3,4-Dihydroxymandelic acid, 4TMS	C ₂₀ H ₄₄ O ₅ Si ₄	476.90 7	0.83
32	14.9 25	Oxazepam, 2TMS derivative	C ₁₅ H ₁₁ ClN ₂ O ₂	286.71 3	0.72
33	15.6 07	Heptasiloxane, hexadecamethyl-	C ₁₆ H ₄₈ O ₆ Si ₇	533.15	0.64
34	16.2 48	Heptasiloxane, hexadecamethyl-	C ₁₆ H ₄₈ O ₆ Si ₇	533.15	0.75

RT: Retention Time (minutes).

4.2: Body Weight

Throughout the study, changes in body weight were recorded for each experimental group. Out of all the groups, the control group had the highest values and maintained a constant body weight. The group that received rotenone, on the other hand, showed a noticeable and statistically significant decrease in body weight, indicating the negative systemic effects of rotenone administration. In comparison to the only rotenone treated group, the co-administration of *Ziziphus jujuba* extract at 200 mg/kg with rotenone moderately improved weight loss.

Body weight values in the rotenone + 400 mg/kg extract group were close to those of the control group, indicating a more noticeable protective effect. It's interesting to note that the 400 mg/kg extract group maintained weights similar to the control group, suggesting that the fruit extract had no negative effects. Although the gain was slightly less than that observed in the high-dose extract group, the rotenone + Sinemet group revealed a significant improvement in body weight when compared to the rotenone group. These findings imply that *Z. jujuba* fruit extract, especially at 400 mg/kg, can counteract weight loss caused by rotenone with effectiveness on the same level with conventional treatment (Fig: 4.2).

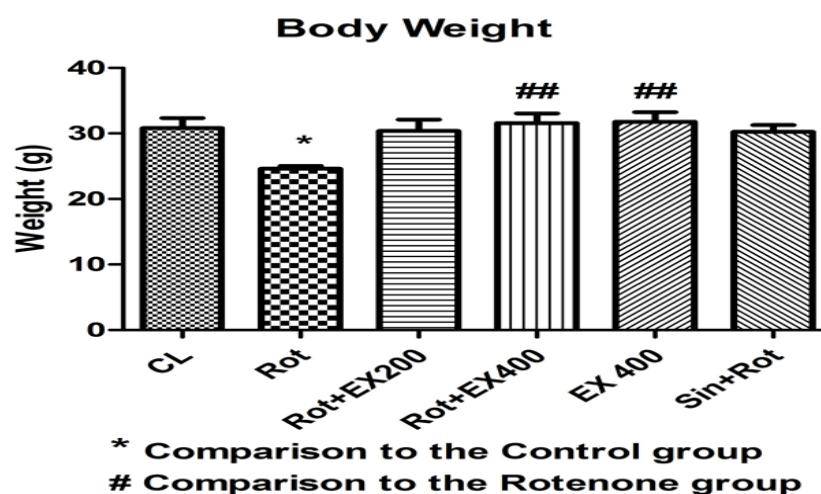


Figure 4.2: Effect of *Z. jujuba* extract administration on rotenone exposed mice. Oneway Anova followed by Bonferroni post-hoc analysis.

4.3: Neurobehavioral Improvements Induced by *Ziziphus jujuba* Extract in Parkinsonian Mice

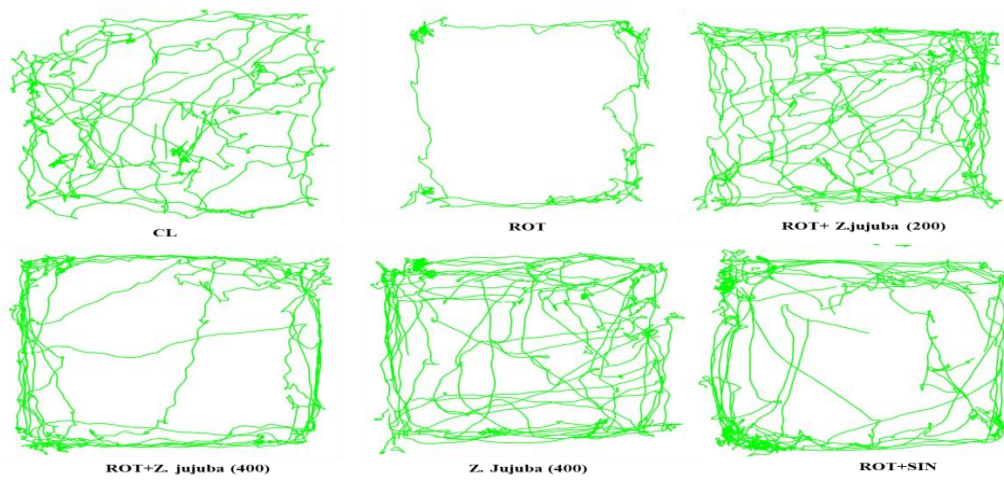
Baseline behavioral tests were done before treatment to confirm that all mice showed similar behavior.

4.3.1: Open Field Test

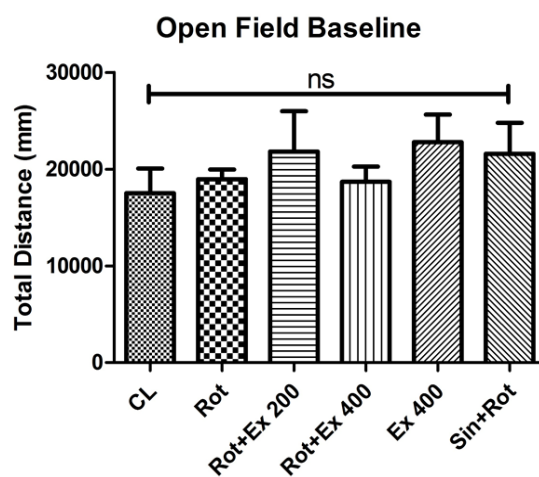
Using ToxTrac software, the locomotor trajectories of mice within the wooden apparatus were analyzed over a 5-minute observation period.

Rotenone treated group ($9209.64 \pm 828.9\text{mm}$) showed significant decrease in locomotor activity comparing to the control group ($16.668.04 \pm 1826.4\text{mm}$). The distance covered by ROT+*Z. jujuba*(200) group ($31688.87 \pm 1457.0\text{mm}$) was highly significant ($p < 0.01$) in comparison to the rotenone treated group. The distance covered by ROT+*Z. jujuba*(400) group ($22909.68 \pm 834.6\text{mm}$), *Z. jujuba*(400) group ($21903.02 \pm 1362.1\text{mm}$) and ROT+Sinemet ($23573.4 \pm 1171.6\text{mm}$) was significant in comparison to rotenone treated group.

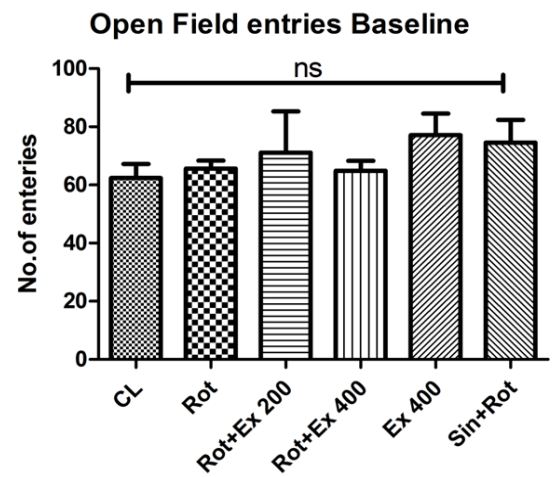
The entries number in rotenone treated group (17 ± 2.9) was significantly different in comparison to control group (60 ± 6.5). The entries number in *Z. jujuba*(200)+ROT was (106 ± 17.26) highly significant ($p < 0.001$) in comparison to control and rotenone treated group. The entries number in *Z. jujuba*(400)+ROT (72 ± 6.64), *Z. jujuba*(400) was (77 ± 13.13) and in ROT+ SIN (78 ± 8.82) was significant ($p < 0.01$) in comparison to rotenone group (Figure:4.31).



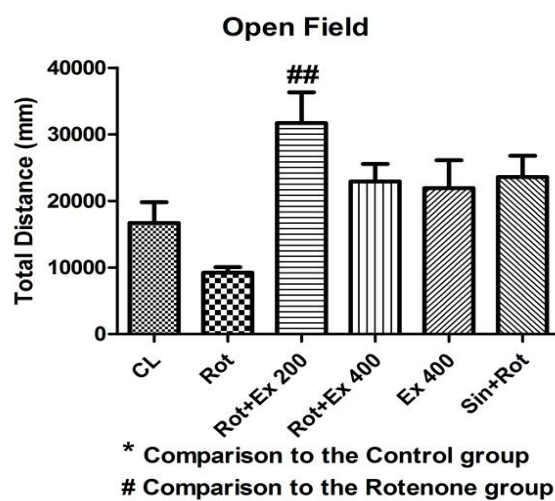
(A)



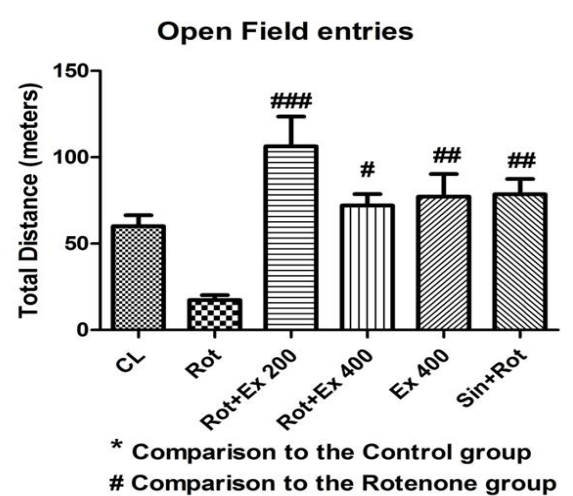
(B)



(C)



(D)



(E)

Figure 4.3.1: *Z. jujuba* extract preserved the locomotory activity after injecting rotenone. (A) Open field trajectories showing the exploratory activity of mice shows that locomotor activity of rotenone treated mice was reduced; in contrast, the *Z. jujuba*(200) +ROT, *Z.jujuba*(400) +ROT, *Z.jujuba*(400) and SIN+ROT treated groups showed increase in locomotor activity. (B) and (C) both showing that all values are non-significant. (D) Distance covered by mice in rotenone treated group was significantly reduced in comparison to control group. In contrast *Z. jujuba*(200) +ROT group marked a significant increase in total distance travelled. While *Z. jujuba*(400) +ROT, *Z. jujuba*(400) and ROT+SIN also showed improvements in distance travelled as compared to rotenone treated group. (D) *Z. jujuba*(200) +ROT, *Z. jujuba*(400) +ROT, *Z. jujuba*(400) and ROT+SIN showed improvement is observed in the reduced entries number into the designated floor area of open field. Data represented as mean $\pm$ SEM; *** p <0.001 vs CL; ## p <0.01 vs ROT. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.3.2: Narrow Beam Walk Test

The narrow beam walk test revealed a major decrease in time taken to travel the 1 m beam in the rotenone induced group in comparison to control group. The average time to travel 1 m length by untreated group (7.75 ± 1.31 s) was significantly (p <0.01) lesser in comparison to rotenone administered group (14.83 ± 2.9 s). Time took to cover the 1 m length in *Z. jujuba*(200)+ ROT group (6.00 ± 0.4 s), *Z.jujuba*(400)+ ROT group (9.00 ± 0.4 s), *Z. jujuba*(400) group (6.5 ± 0.8 s), and SIN+ROT group (8.8 ± 1.5 s) significantly lesser in comparison to rotenone administered group (Figure 4.3.2).

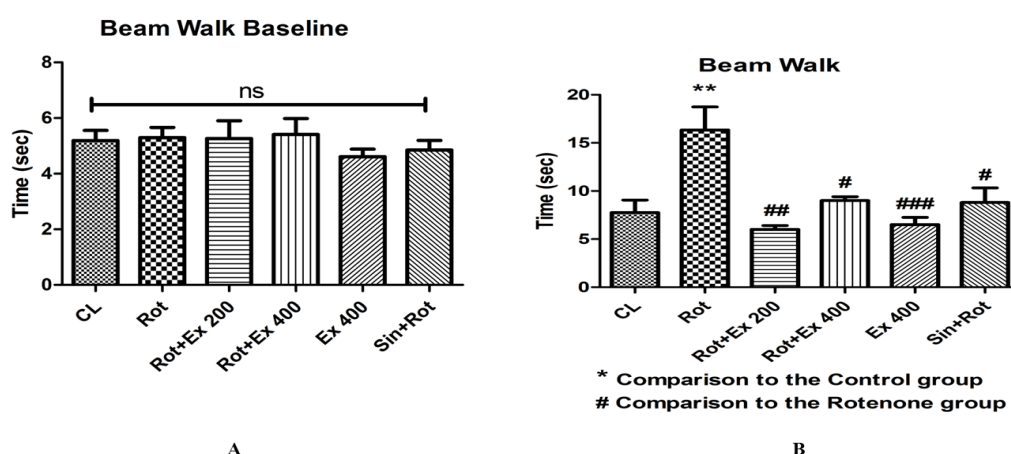


Figure 4.3.2: (A): Before dosing all values are non-significant. (B): A significant decrease in time to cross 1 m distance in *Z. jujuba*(200)+ ROT, *Z. jujuba*(400)=ROT, *Z. jujuba*(400)+ROT and SIN+ROT is seen in comparison to rotenone induced group. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.3.3: Pole Climb down Test

An increase in time taken to descend the pole was monitored in rotenone induced mice ($14.83 \pm 1.62s$) when compared with control group ($7.25 \pm 0.25s$). Total time taken to climb down pole in *Z. jujuba*(200)+ROT group ($6.00 \pm 0.41s$), *Z. jujuba*(400)+ROT group ($7.75 \pm 1.03s$), *Z. jujuba*(400) group ($7.00 \pm 0.45s$) and SIN+ROT group ($7.8 \pm 0.38s$) were significantly ($p < 0.001$) reduced in comparison to rotenone treated mice. Rotenone administration resulted in a significant ($p < 0.001$) increase in time required to descend the pole ($0.98 \pm 0.03s$) as compared to control group ($0.24 \pm 0.02s$). However the time was (0.25 ± 0.03) in *Z. jujuba*(200)+ROT, (0.23 ± 0.01) in *Z. jujuba*(400)+ROT, (0.21 ± 0.02) in *Z. jujuba*(400), and (0.27 ± 0.01) groups showed the significant ($p < 0.001$) preservation of locomotor ability (Figure 4.3.3).

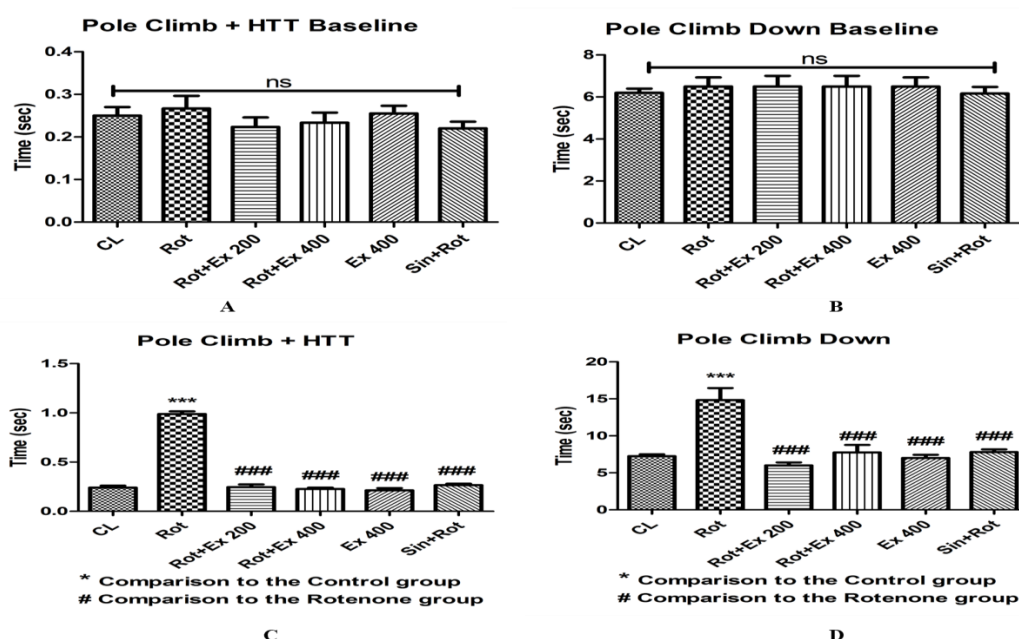


Figure 4.3.3: (A) and (B) both showing that values of head turn time and time taken to descend down are non-significant.(C) An increase in time taken by rotenone treated group as compared to other treatment groups. (D) *Z. jujuba*(200)+ROT group, *Z. jujuba*(400)+ROT group, *Z. jujuba*(400) group and SIN+ROT group showed decrease in time to climb down in comparison to rotenone treated group. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.3.4: Stride length Test

Stride length measurements were 6.03 ± 0.25 cm for mice in untreated group, 4.94 ± 0.07 cm for rotenone induced group, 5.56 ± 0.27 cm for *Z. jujuba*(200)+ ROT group, 5.33 ± 0.10 cm for *Z. jujuba*(400)+ ROT group, 6.19 ± 0.25 cm for *Z. jujuba*(400) group and 6.78 ± 0.26 cm for SIN+ROT group. Shorter strides were significantly observed after rotenone administration while recovery was observed in *Z. jujuba*(200)+ROT, *Z.jujuba*(400)+ ROT, *Z.jujuba*(400)+ ROT and SIN+ROT groups (Figure 4.3.4).

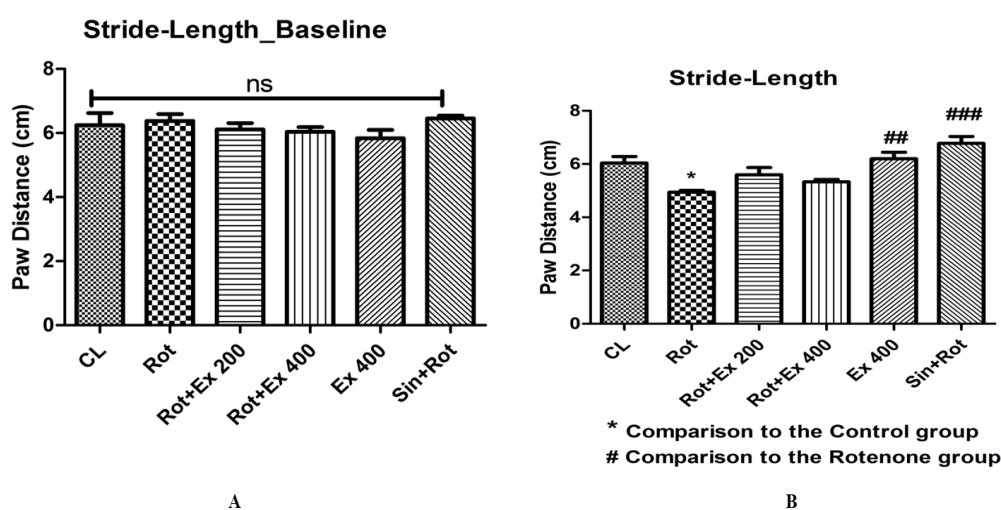


Figure 4.3.4: (A) All values are non-significant. (B) Decrease in stride length in rotenone treated group in comparison to control group and was rescued in all other treatment groups. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.3.5: Tail suspension Test

The duration of immobility time while suspended by tail was 104.75 ± 10.3 s in control group, 218.83 ± 23.1 s in rotenone treated group, 97.5 ± 14.0 s in *Z. jujuba*(200)+ ROT group, 180.00 ± 7.4 s in *Z. jujuba*(400)+ ROT group, 114.33 ± 22.4 s in *Z. jujuba*(400)= ROT group and 161.5 ± 27.0 s in SIN+ROT group. The immobility time in rotenone was significantly ($p < 0.01$) increased in comparison to untreated group, while *Z. jujuba*(200)+ROT and *Z. jujuba*(400) significantly rescued the immobility time. *Z. jujuba*(400)+ ROT and SIN+ROT groups were not quite different from rotenone treated group (Figure 4.3.5).

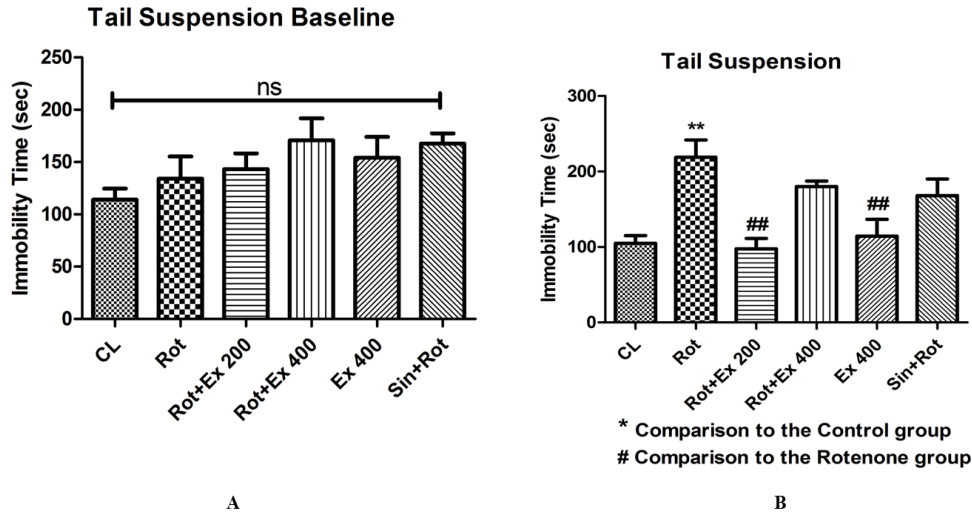


Figure 4.3.5: (A) All values are non-significant. (B) An increase in immobility time as compared to control group. While immobility time is rescued in *Z. jujuba*(200)+ ROT and *Z. jujuba*(400) group. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.3.6: Stepping Test

The time took to cover the distance in straight corridor of 1m length was significantly increased ($p < 0.001$) in rotenone induced mice (19.5 ± 1.1 s) as compared to control group (8.00 ± 0.71 s) indicating reduced movement speed. The time taken to cross same corridor by *Z. jujuba*(200)+ ROT group (6.75 ± 0.63 s), *Z. jujuba*(400)+ ROT group (7.25 ± 0.63 s), *Z. jujuba*(400) group (8.33 ± 0.33 s) and SIN+ROT group (8.8 ± 1.4 s) which marked the significant ($p < 0.001$) decrease in time in comparison to rotenone treated group (Figure 4.3.6).

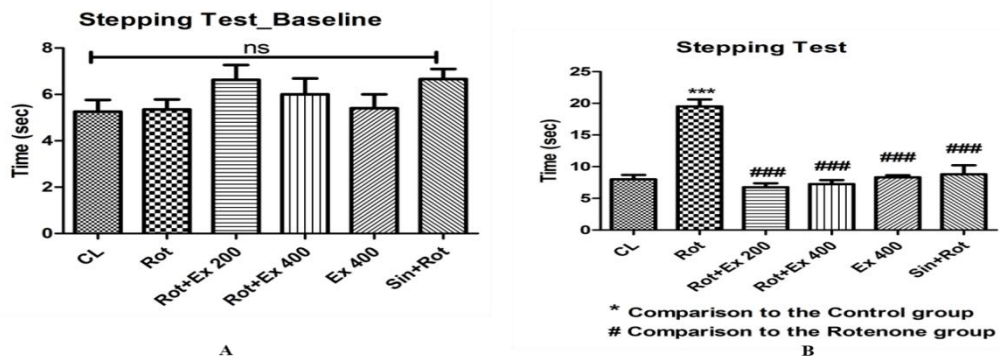


Figure 4.3.6: (A) All values are non-significant. (B) Increase in time taken by rotenone treated group while improvement in time taken by all other treatment groups. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.4: Histopathological Examination of Midbrain Section

All experimental groups had coronal slices of the midbrain that included the substantia nigra area histologically analyzed after 21 days of rotenone (2.5 mg/kg subcutaneously) and *Z. jujuba* extract (200-400 mg/kg orally). The cellular architecture of this area varied noticeably amongst the groups (Fig: 4.4.A).

4.4.1: Control Group

Histological examination of the midbrain in mice in the control group showed that the substantia nigra's cytoarchitecture was well-preserved, with intact, closely spaced dopaminergic neurons. There were no indications of gliosis, vacuolization, or neuronal loss, suggesting adequate neuroanatomical integrity. A normal amount of multipolar cells are present (Fig: 4.4.B).

4.4.2: Rotenone treated Group

Significant neuronal death in the substantia nigra, vacuolization, and decreased cellular density were among the notable neurodegenerative alterations seen in the rotenone-treated group. There were a few of shrunken, darkly pigmented neurons that showed signs of neuronal degeneration. The increasing number of glial nuclei indicated gliosis in the surrounding tissue. Neurodegeneration resulted in the large number of vacuolated cells (Fig: 4.4.C).

4.4.3: *Z. jujuba* (200) + ROT Group

Histological examination of the substantia nigra in the midbrain revealed undamaged, well-spaced neurons with large number of multipolar cells indicating no evidence of gliosis or degeneration. A healthy area of the brain is indicated by normal cytoarchitecture (Fig: 4.4.D).

4.4.4: *Z. jujuba* (400) + ROT Group

The substantia nigra area of the midbrain slice has comparatively intact neuronal architecture. Neurons show fewer obvious evidence of degeneration and seem fairly thick. In comparison to the PD group, the cytoarchitecture appears to have improved, with minute amount of vacuolated cells indicating some neuroprotection that may

have resulted from therapy. There is no discernible vacuolization or gliosis (Fig: 4.4.E)

4.4.5: *Z. jujuba* (400) Group

The neurons in this region are not damaged. There is no sign of degeneration. There is no any vacuolization or gliosis. Neurons are in dense quantity and are not shrunken in size. The multipolar cells are present just like the control group. It revealed that extract has not any side effect on region of substantia nigra (Fig: 4.4.F).

4.4.6: Sinemet + ROT Group

It showed the clear and abundant density of neurons. But there are some sign of neurons shrinkage with small number of vacuolated cells. But they are not much as like in rotenone treated group.

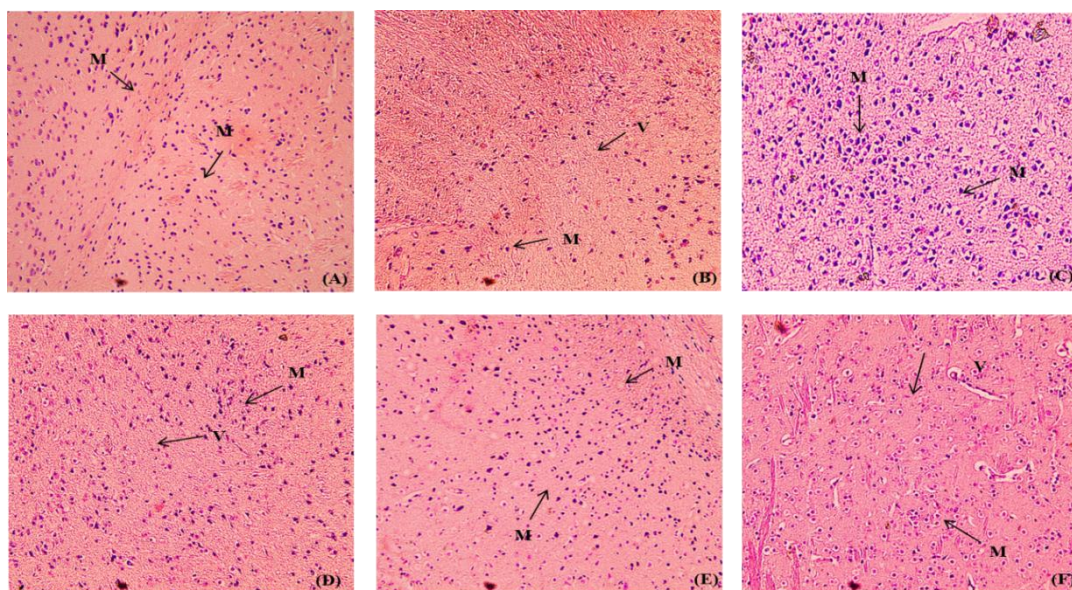


Figure 4.4: (A): Control group showing normal multipolar cells. (B): Lesser number of multipolar cells and more vacuolation in rotenone treated group. (C) *Z. jujuba*+ ROT treated group showed lesser vacuolation and prevented multipolar cells. (D): Higher number of multipolar cells but somewhere vacuolation is present. (E): *Z. jujuba* extract treated group showed the health amount of multipolar cells just like control group. (F): Multipolar cells are present but it showed some vacuolation here.

4.5: Impact of *Zizyphus jujuba* Extract on Oxidative stress Associated Genes

The expression levels of important antioxidant and cytoprotective genes were assessed in the substantia nigra using quantitative RT-PCR in order to better understand the molecular processes behind the neuroprotective effects of *Zizyphus jujuba* ethanolic extract. Among the genes that were chosen were SOD2, Nrf2, HO-1, Txnrd1, and MT1, all of which are essential for controlling oxidative stress, redox homeostasis, and neuronal survival. The experimental groups had distinct expression patterns, underscoring the extract's modulatory effect on transcriptional responses linked to the pathophysiology of PD.

4.5.1: Metallothionein-1 (MT1)

A metal-binding protein called metallothionein-1 (MT1) is crucial for detoxifying heavy metals, preserving cellular redox balance, and shielding neurons from oxidative damage. MT1 expression is especially important in the substantia nigra because it helps protect neurons in the heightened oxidative stressors found in PD. MT1 mRNA levels were measured in the experimental groups to evaluate its regulation.

The experimental groups did not differ in any statically significant ways according to analysis of MT1 gene expression. The rotenone-treated group showed a slight increase in MT1 mRNA levels, by comparing to the control group which could indicate a compensatory stress response. However, MT1 expression did not significantly change after treatment with *Zizyphus jujuba* ethanolic extract by comparing to the control or rotenone groups ($p > 0.05$), suggesting that the intervention had no effect on MT1. A slight deviation in gene expression was observed in Sinemet treated group in comparison to rotenone and extract treated group (Fig: 4.5.1).

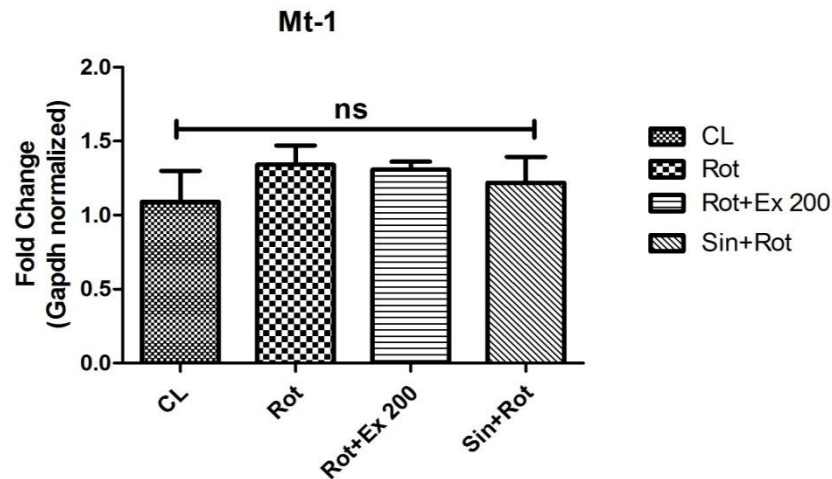


Figure. 4.5.1: Mt-1 gene showing that its expression is non- significant in all groups. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.5.2: Superoxide Dismutase 2 (SOD 2)

Superoxide Dismutase 2 is an antioxidant enzyme located in the mitochondria that helps in catalyzing the transformation of superoxide radicals into hydrogen peroxide and molecular oxygen, serving as the body's first line of defense towards oxidative stress. Changes in SOD2 expression within the substantia nigra offer crucial insights into neuronal vulnerability because mitochondrial dysfunction and elevated reactive oxygen species are key components of Parkinson's disease pathology. SOD2 mRNA levels in the experimental groups were measured.

When comparing the rotenone-treated group to the controls, qRT-PCR analysis of SOD2 showed a slight increase, but this difference was not statistically significant ($p > 0.05$). The extract-treated group exhibited a similar expression pattern and did not significantly different from the rotenone treated group. The Sinemet treated group marked a slight deviation in gene expression in comparison to rotenone and extract treated group. These results indicate that *Zizyphus jujuba* ethanolic extract did not significantly affect SOD2 expression under these circumstances, even though rotenone exposure may cause a compensatory increase in mitochondrial antioxidant defense.

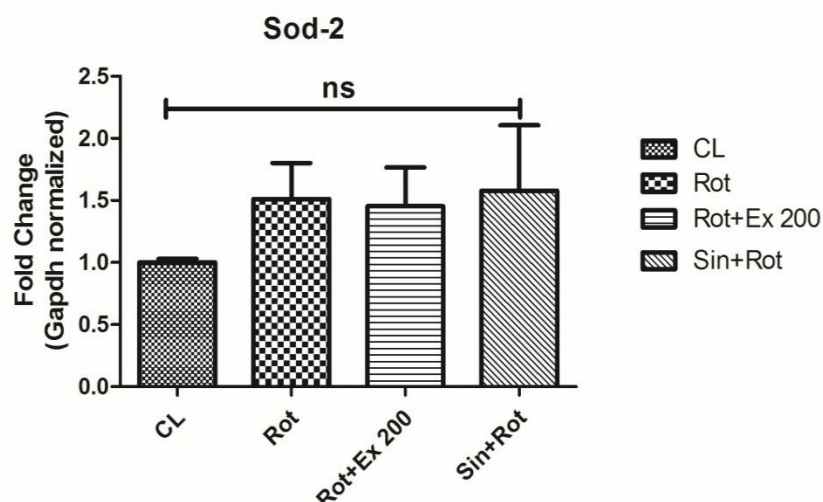


Figure. 4.5.2: Sod-2 gene showing that its expression is non-significant in all treated groups with slight deviation among bars. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.5.3: Nuclear factor erythroid 2–related factor 2 (Nrf2):

A crucial transcription factor called nuclear factor erythroid 2–related factor 2 stimulates the expression of cytoprotective genes like HO-1, SOD2, and Txnrd1, which in turn controls the cellular antioxidant response. To preserve redox balance in the face of oxidative stress, Nrf2 separates from its inhibitor Keap1 and moves into the nucleus, where it activates antioxidant response elements (AREs). Changes in Nrf2 expression in the substantia nigra are important for comprehending the molecular mechanisms underlying Parkinson's disease because of its critical function in preventing oxidative damage. In order to assess this, the experimental groups' Nrf2 mRNA levels were measured.

The rotenone-treated group's Nrf2 gene expression was higher than that of the controls, suggesting that antioxidant signaling was activated against oxidative stress. It's interesting to check that the extract-treated group showed a marginally lower expression level than the rotenone group, but the difference was not statistically significant ($p > 0.05$). A slight deviation in gene expression was analyzed in Sinemet treated group in comparison to rotenone treated group but showed same expression like extract treated group. These results indicate that although rotenone caused Nrf2 to be upregulated in a compensatory manner, *Zizyphus jujuba* ethanolic extract did not increase Nrf2 expression any further under the current experimental setup (Fig: 4.5.3).

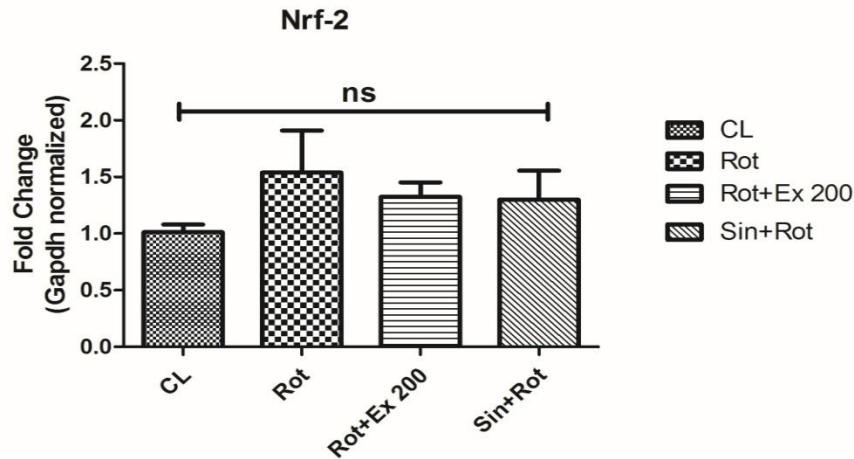


Figure. 4.5.3: Nrf-2 gene expression is non-significant with a slight deviation among bars in all treated groups. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.5.4: Thioredoxin reductase-1

A selenoprotein enzyme called thioredoxin reductase 1 (Txnrd1) is essential to the thioredoxin system, which reduces oxidized thioredoxin to preserve cellular redox homeostasis. Txnrd1 supports DNA synthesis and restoration, antioxidant defense, and defense against oxidative stress-induced apoptosis by using this mechanism. In neurodegenerative illnesses like Parkinson's disease, neuronal impairment has been connected to Txnrd1 dysregulation. Txnrd1 mRNA levels within the substantia nigra of experimental groups were measured to assess its role in the current study.

Txnrd1 gene expression was reduced in the rotenone treatment group in comparison to the untreated group according to quantitative analysis, suggesting that the thioredoxin system is not functioning as well under oxidative stress. Surprisingly, the *Zizyphus jujuba* ethanolic extract treatment substantially raised the Txnrd1 expression, exceeding both the rotenone and control groups. The Sinemet treated group marked an increase in gene expression in comparison to rotenone treated group but no more significance from extract treated group. This increase implies that the extract significantly triggered the thioredoxin antioxidant pathway, which in turn helped to improve substantia nigra cellular defense (Fig: 4.5.4).

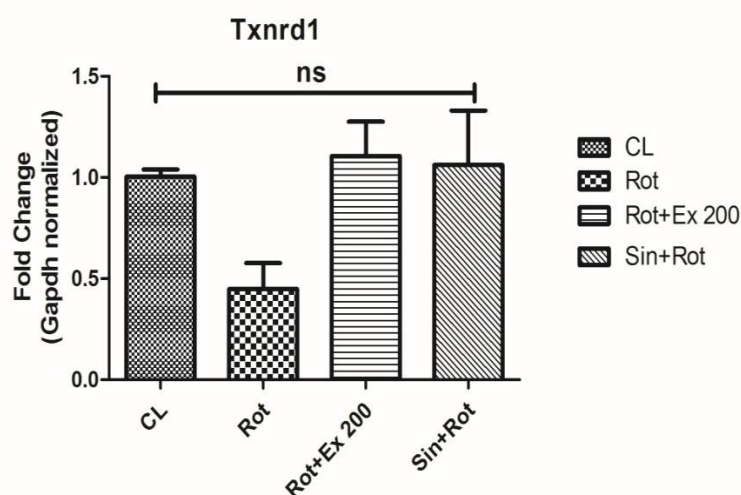


Figure. 4.5.4: Txnrd1 gene showing its expression none significant with deviation among bars in all treated groups. Oneway ANOVA followed by Bonferroni post-hoc analysis.

4.5.5: Heme oxygenase-1 (Hmox1)

The stress-triggered enzyme heme oxygenase-1 regulates the dissociation of heme into free iron, carbon monoxide, and biliverdin. Apart from its function in heme catabolism, HO-1 is well known for being an anti-oxidant and anti-inflammatory enzyme that works downstream of Nrf2 signaling to reduce inflammatory processes and oxidative stress in cells. PD development is believed to be linked to imbalances of expression of HO-1 in the substantia nigra, which represents the neuronal adaptive response to oxidative damage. The results of Hmox1 mRNA levels were measured in the substantia nigra of the experimental groups to evaluate its involvement.

While comparing to the untreated group, the rotenone group's HO-1 expression analysis showed a non-significant expression. Curiously, HO-1 mRNA levels were further increased by *Zizyphus jujuba* ethanolic extract treatment above those seen in the rotenone group but still its expression is non-significant here. While the Sinemet treated group showed no significance as compared to rotenone treated group. This significant upregulation suggests that the extract strongly activates the Nrf2/HO-1 axis, underscoring its potential to strengthen the substantia nigra's natural antioxidant defenses (Fig: 4.5.5).

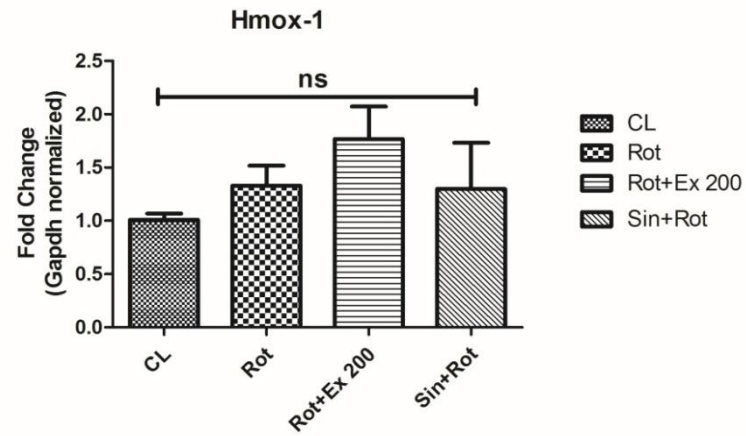


Figure. 4.5.5: Hmox-1 gene expression is non- significant in all groups with deviation among bars in all treated groups. Oneway ANOVA followed by Bonferroni post-hoc analysis.

CHAPTER NUMBER 5

Discussion

Natural substances with high neuroprotective and antioxidant qualities have drawn a lot of interest as possible PD treatments. The current study revealed the neuroprotective potential of an ethanolic extract from *Ziziphus jujuba* fruit against Parkinson's disease using an animal model induced by rotenone. Numerous bioactive constituents, such as flavonoids, phenolic acids, alkaloids, saponins, and fatty acid derivatives, were discovered after the ethanolic extract was subjected to GCMS analysis. These Phytoconstituents well-established neuromodulatory, anti-inflammatory, and antioxidant properties and could account for the favorable results of this study. Ethanolic extraction in particular is considered to produce a wide variety of polyphenols and saponins when compared to aqueous preparations, which makes it a suitable solvent solution for separating neuroprotective compounds from *Z. jujuba* [86].

Behavioral assessments revealed that rotenone-treated mice exhibited severe motor impairments, reduced locomotor activity, and poor coordination. These findings are aligned with early literature revealing that rotenone impacts mitochondrial complex I, leading to dopamine producing neurons in the substantia nigra dying. Treatment with ethanolic *Z. jujuba* extract significantly improved motor performance, suggesting that dopaminergic neuronal function was maintained. This improvement may be due to the antioxidative and anti-apoptotic qualities of ethanol-extracted flavonoids, such as rutin, quercetin, and kaempferol, which have been identified in *Z. jujuba* and are known to protect against neurotoxin induced dopaminergic cell death [120].

The behavioral findings were validated by histological examination of midbrain sections. Groups treated with rotenone showed signs of neuronal shrinkage, vacuolation, and loss of pigmented dopamine producing neurons, all of which are hallmark features of PD pathology. In contrast, the mice given ethanolic extract from *Z. jujuba* showed improved cellular integrity, less neuronal degeneration, and the preservation of substantia nigra neurons. Ethanol-soluble jujube compounds' capacity to scavenge free radicals and reduce lipid peroxidation, thereby averting oxidative stress-induced neurodegeneration, is responsible for these effects.

Ethanollic extracts of *Z. jujuba* have demonstrated neuroprotective benefits in ischemia and neurotoxicity models by activating Nrf2 signaling and boosting endogenous antioxidant defenses, according to similar findings from earlier studies [121].

Using qPCR for molecular analysis, more mechanistic insights were discovered. Pro-oxidant markers were upregulated and cytoprotective genes were downregulated in the rotenone group, indicating significant oxidative stress and neuroprotective gene dysregulation. Following treatment with ethanollic *Z. jujuba* extract, the expression of the genes Sod2, Txnrd1, Mt1, and Hmox1 and Nrf2 returned to normal, suggesting that the extract not only reduces oxidative damage but also activates transcriptional programs related to cellular defense and redox equilibrium. According to earlier research, *Z. jujuba* ethanollic extracts enhance neuronal survival by activating the Nrf2/ARE pathway, which raises the expression of detoxifying enzymes like HO-1, NQO1, and thioredoxin reductase [122].

The neuroprotective effect observed in this study may be due to the anti-inflammatory characteristics of *Z. jujuba* ethanollic extract. Pro-inflammatory cytokine production and microglial activation mediate chronic neuroinflammation, which is the primary causes of PD development. Ethanol fractions from *Z. jujuba* have been shown in experimental models to inhibit NF- κ B activation and reduce inflammatory mediators such as TNF- α , IL-1 β , and COX-2. By reducing neuroinflammation, the extract most likely supported behavioral recovery and preserved dopaminergic neurons in this study [123].

Overall, the study's findings demonstrate that ethanollic *Ziziphus jujuba* fruit extract offers a strong defense against rotenone-induced Parkinsonism. The three primary elements of the protective processes appear to be enhanced neuroprotective gene production, antioxidant defense, and anti-inflammatory activity. These findings, which are in line with earlier experimental studies on jujube and related medicinal plants, demonstrate the therapeutic efficacy of ethanol extracted phytochemicals in neuroprogressive disorders. These preclinical results are extremely encouraging, but further research is needed to determine specific active ingredients, analyze dose-response relationships, and evaluate long term efficacy in models of chronic Parkinson's disease. Clinical research would eventually be required to validate the translational value of *Z. jujuba* ethanollic extract for the treatment of PD humans.

CHAPTER NUMBER 6

Conclusion

The current research gives the robust evidence that the ethanolic extract of *Ziziphus jujuba* fruit contains the significant neuroprotective qualities in a rotenone-induced model of PD. Several bioactive phytochemicals with neuromodulatory, anti-inflammatory, and antioxidant properties were found by the extract's comprehensive GC-MS profiling. According to behavioral assessments, giving *Z. jujuba* extract to ill mice improved their locomotor function and considerably decreased their motor deficits.

Histopathological analysis further showed that the extract maintained the structural integrity of dopamine producing neurons in the striatum and substantia nigra, two areas that are severely impacted in PD. Furthermore, qPCR analysis revealed that the cytoprotective and antioxidant genes were positively regulated, indicating that the extract strengthens the body's defenses against oxidative stress and neuronal death.

Together, the outcomes of this study indicated that *Z. jujuba* fruit extract offers significant neuroprotection against rotenone-induced Parkinson's disease by improving motor coordination and reducing behavioral abnormalities. It preserves neuronal structure and prevents histological degradation. It is helpful in boosting the expression of antioxidant genes and neurotrophic support at the molecular level. Also it fights against oxidative stress and inflammation that are two important mechanisms in the pathophysiology of Parkinson's disease.

Therefore, *Z. jujuba* exhibits promise as a natural treatment with a range of neuroprotective properties. Its efficacy seems to be regulated by antioxidant and anti-inflammatory pathways in addition to the regulation of neurotrophic factors. Further research is required to determine the exact active ingredients, optimize dosage, and verify the extract's long-term safety and therapeutic suitability.

In conclusion, this thesis provides substantial preclinical evidence supporting the potential application of *Ziziphus jujuba* fruit extract as an adjuvant therapy strategy for PD. By connecting the dots between phytochemical richness and functional neuroprotection, the study contributes to the growing body of evidence showing the effectiveness of medicinal plants as weapons in the fight against neurodegenerative diseases.

CHAPTER NUMBER 7

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