STUDIES ON ROTENONE INDUCED NEURODEGENERATION IN *DROSOPHILA MELANOGASTER* AND ITS MODULATION THROUGH GREEN ENGINEERED NANOPARTICLES

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DECLARATION

I, hereby declared that the presented work in the thesis entitled "Studies on Rotenone induced neurodegeneration in *Drosophila melanogaster* and its modulation through green engineered nanoparticles" in fulfilment of degree of **Doctor of Philosophy (Ph.D.)** is outcome of research work carried out by me under the joint supervision of ***Dr. Joydeep Dutta***, working as Professor and Head, in the Department of Zoology, School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India and ***Dr. Mahendra Pratap Singh,*** Associate Professor in the Department of Zoology, DDU Gorakhpur University, Gorakhpur, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.

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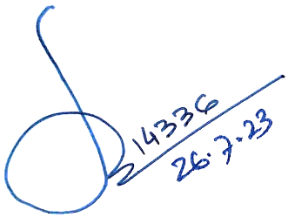
CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled **Studies on Rotenone induced neurodegeneration in *Drosophila melanogaster* and its modulation through green engineered nanoparticles** submitted in fulfillment of the requirement for the reward of degree of **Doctor of Philosophy (Ph.D.)** in **Zoology, School of Bioengineering and Biosciences**, is a research work carried out by **Shabnam Shabir, 11919620**, is bonafide record of her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.



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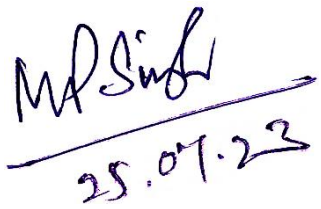

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ABSTRACT

Neurodegenerative disorders are primarily caused by the accumulation of abnormal aggregates of protein in the brain, leading to the malfunctioning and eventual death of neurons. Systemic administration of drug to the CNS is a substantial obstacle, owing to their short half-life, considerable first-pass digestion, restricted accessibility to the brain, and potential adverse impact when accessing non-target peripheral organs. As a result, developing systemic delivery mechanisms with higher potency is critical for CNS pharmacotherapy. Cholinesterase (ChE) inhibitors, tacrine, N-methyl-D-aspartate (NMDA) agonists in connection with Vitamin D, levodopa, or other dopaminergic agonists and memantine have never been utilized in conventional medicine or therapeutics to alleviate anything apart from motor symptoms by replenishing neurotransmitters. However, prolonged use of these medications can have substantial side effects, including other motor challenges. To stop or reduce the progression of numerous neurodegenerative disorders, researchers must create new natural neuroprotective agents due to the inadequacy of treatment medicines. By activating the Nrf2/Akt/PI3K cascade and eliminating free radicals, nutraceuticals and other phytonutrients have been proven to have protective benefits and to reduce the consequences of neurodegenerative conditions. The Ayurvedic medicinal system has long employed the plants UD (*Urtica dioica*), MC (*Matricaria chamomilla*), and MK (*Murraya koenigii*) as well-known nerve relaxants and cognition boosters. These three herbs include phytochemicals with proven protective properties, including quercetin, alkaloids, vitamins, and polyphenols. Drug targeting and delivery of dietary antioxidants to the brain provides significant obstacles due to the BBB in treating oxidative stress-related disorders, particularly neurodegenerative diseases. Novel approaches for the improved crossing of the BBB must be developed in order to progress in the effective therapies. Nanotechnology holds significant ameliorative potential against neurodegenerative diseases as it can protect the therapeutic substance and allow for its sustained release. This study demonstrated that pure zinc oxide nanoparticles (ZnO-NPs) of magnificent quality were fabricated using zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) as the precursor and plant UD, MC, and MK fractions as the reducing agents against ROT-induced toxicity in *Drosophila melanogaster* wild-type Oregon R^+ at cellular, neurochemical, biochemical, behavioral, and molecular levels for the first time. Plant extracts (ethanolic and aqueous) were produced from all three plants UD, MC, and MK, and their bioactive elements were determined using cell free models (ABTS, DPPH, TFC, and TPC). To determine the wavelengths at which plant compounds absorb light and measure their concentration based on the absorption intensity, UV-

Vis, FT-IR, and HPLC techniques were used. By using green method UD-ZnO, MC-ZnO, and MK-ZnO nanoparticles were synthesized. The optical and structural properties of biosynthesized ZnO-NPs were analysed through FT-IR, DLS, XRD, EDS, SEM, UV-Vis, and Zeta potential. The antimicrobial properties of fabricated ZnO-NPs were evaluated employing well diffusion method in bacteria (*Staphylococcus aureus* and *Escherichia coli*). The rotenone (500 µM) was administered to *Drosophila* third instar larvae, and freshly emerged flies for 24-120 hours, either alone or in combination with plant extracts and their biogenic ZnO nanoparticles. Comparative research on the protective efficacy of synthesized NPs was undertaken against rotenone-induced toxicities. Following exposure, dye exclusion assay (cytotoxicity test), neurochemical analyses (acetylcholinesterase and butrylcholinesterase inhibition assessments), biochemical assays such as catalase (CAT), superoxide dismutase (SOD), protein carbonyl (PC), lipid peroxidation (LPO), total protein estimation, and glutathione reductase (GSH), and behavioral assays (climbing, memory, jumping and crawling assays). To investigate whether there is any alteration in the shape or size of the *Drosophila* eyes treated with rotenone, eye imaging under stereo zoom microscope was done. To further understand the underlying mechanisms of rotenone-induced degeneration, we performed molecular analyses, such as gene expression profiling (RT-PCR), to identify changes in specific genes (*APP, tau,* and *α-Syn*) or signalling pathways associated with the degenerative process. The results revealed that MK possessed the strongest antioxidant properties among all three plant extracts due to the greatest TFC, TPC, ABTS, and DPPH levels, subsequent to UD, and lastly MC. UV-Vis spectroscopy recorded valuable information about the presence and concentration of various compounds such as pigments, flavonoids, phenolics, and other bioactive molecules in the plant extracts. FT-IR spectroscopy provided valuable information about the chemical composition (such as carbohydrates, proteins, lipids, phenolics, and other bioactive molecules), functional groups C-H, O-H, C-O, N=C, C=N, N-H, C-N, C-C, C=O, and S=O, and structural characteristics of plant extracts. HPLC analysis of plant extracts provides valuable information about the presence, composition, and concentration of various secondary metabolites such as alkaloids, flavonoids, phenolics, terpenoids, and other secondary metabolites in plant extracts. The ZnO-NPs were biosynthesized in the form of pale yellow paste, which were calcinated at 400˚C to form powder and used for characterization. By utilizing FT-IR, DLS, XRD, EDS, SEM, UV-Vis, and Zeta potential techniques, the findings indicated that the NPs exhibited agglomerated crystalline structures with a hexagonal shape and an average diameter of 20-40 nm. After characterization these biogenic ZnO-NPs were used for *in vivo* studies. Among the plant derived ZnO-NPs, MK-ZnO NPs exhibit strong anti-

microbial activity to damage cell membrane structures and permeability barriers with loss of chemiosmotic control followed by UD-ZnO NPs, and then MC-ZnO NPs. In this regard, ethno-nano medicinal therapeutic uses mimic the similar effects in *D. melanogaster* by suppressing $p<0.05$ oxidative damage and counteracting the inhibitory effect of rotenone on neurochemical activities AChE and BChE activity. MK-ZnO NPs showed the enhanced protective effects against oxidative stress by restoring biochemical parameters CAT, SOD, LPO, PC, GSH and proteotoxicity activity followed by UD-ZnO and then MC-ZnO NPs. By reducing cellular toxicity (trypan blue), these nanoparticles possess intrinsic antioxidant properties and can help mitigate the generation of ROS. ZnO-NPs effectively protected *Drosophila* from deteriorating locomotor dysfunctions, indicating that they might protect by replenishing the mitochondrial dopamine pool. These nanoparticles modulated and enhanced neuromuscular function, improved muscle strength, coordination, supporting memory processes, and overall climbing performance. By stimulating cellular proliferation, migration, and differentiation, ZnO-NPs may contribute to the restoration of damaged tissues and the recovery of visual function. RT-PCR gene expression studies was performed using *GAPDH* (159 bp) as the housekeeping gene, whereas *APP* (215 bp), *Tau* (105 bp), and *α-Syn* (164 bp) as the target genes. In case of *APP* gene, the maximum ($P<0.05$) upregulation was observed in larvae exposed with ROT in comparison to control, DMSO, and zinc nitrate, respectively. Among the biosynthesized ZnO-NPs groups, ROT plus MK-ZnO NPs exhibits the minimum induction, subsequent to ROT plus UD-ZnO and lastly ROT plus MC-ZnO also showed the decrease in gene expression. Plant extracts such as ROT plus UD, ROT plus MC, and ROT plus MK suppressed the *APP* mRNA expression as well. The same trend was observed in *tau* and *α-Syn* gene i.e. MK-ZnO> UD-ZnO> MC-ZnO NPs. Biosynthesized ZnO-NPs have demonstrated tremendous effects in modulating the aggregates of *APP*, *tau*, and *α-Syn* expressions, which are involved in neurodegenerative pathologies. Nanotherapeutically, the administration of biogenic ZnO-NPs to *Drosophila* could mitigate oxidative stress due to its antioxidative attributes as well as ability to modulate antioxidant defenses. Therefore, the utilisation of nanotechnology to produce non-invasive drug delivery systems may result in the development of innovative and enhanced formulations to facilitate the delivery of therapeutic substances across the BBB and could be used as great alternative for clinical development.

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SHABNAM SHABIR

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INTRODUCTION

Neurodegenerative diseases (NDs) are among the prominent reasons of disability and mortality globally (Borumandnia et al., 2022). The World Health Organization (WHO) has proclaimed it to be the most serious global health hazard of the twenty-first century (Makdissi et al., 2023). Neurodegenerative diseases encompass a broad spectrum of conditions caused by gradual destruction of neurons, neural networks and the glial cells in the brain. That are required for coordination, movement, strength, cognition, and sensation (Kabir et al., 2022). With an ageing global population, the incidence and prevalence of NDs are dramatically growing, providing a challenge to the sustainability of health systems, notably in middle- and low-income nations (Kitchen et al., 2017). In India, it is estimated that 36 million individuals suffer from neurodegenerative disorders out of a population of 1.38 billion (Jin et al., 2023). In accordance to a recent WHO report titled Neurological Disorders, Public Health Challenges, over one billion people worldwide suffer from neurological conditions, and 6.8 million people die from such disorders each year (Alvi et al., 2022). In 2015, the Lancet published a report on the Global Burden of Disease (GBD) project, which stated that NDs account for 16.8 percent of global deaths (GBD, 2022). NDs are incurable and debilitating disorders that are growing more common as the world population ages (Choudhary et al., 2021).

The homeostasis among both antioxidants and oxidants regulates the redox status of cells. The oxidative status of the cell is determined by any imbalance between these two that could lead to necrosis or apoptosis (Kuznetsov et al., 2022). The key factor contributing to the increased oxidative stress sensitivity of brain cells is reactive oxygen species (ROS). Although oxygen (O_2) is typically a non-reactive molecule, it can be metabolized to form oxidation products such as superoxide anions ($O2^-$), hydroxyl radicals (OH^-), and several other reactive species in the body. It leads to dreaded conditions including neurological diseases (Zhou et al., 2022).

NDs are a group of diseases that are typically caused by the axonal transport obstruction, misfolded protein aggregation, dysfunction in the mitochondria, progressive neuronal loss, and neuroinflammation (Wilson et al., 2023). Several neurodegenerative disorders, including, Motor sclerosis, Parkinson's disease, Amyotrophic lateral sclerosis, Alzheimer's disease, and Huntington's disease (Figure 1.1), are caused by ACETYL CH inhibition and rely heavily on free radical species for disease development (Jeromin and Bowser, 2017). The high oxygen consumption needed to meet the brain's high energy requirements specifically leads to excessive ROS production, Inadequate antioxidant defence systems and large quantities of

PUFAs in the membranes of neurons render them more sensitive to oxidation (Singh et al., 2019). Cellular defence systems that include endogenous antioxidant properties and antioxidant enzymes including lipid peroxidase, catalase, glutathione reductase, and glutathione contribute in detoxification (Singh et al., 2022). Significant cell biomolecules such as lipid, DNA, and protein are damaged by the degradation of these defence mechanisms, which ultimately leads to death of cell (Liu et al., 2022).

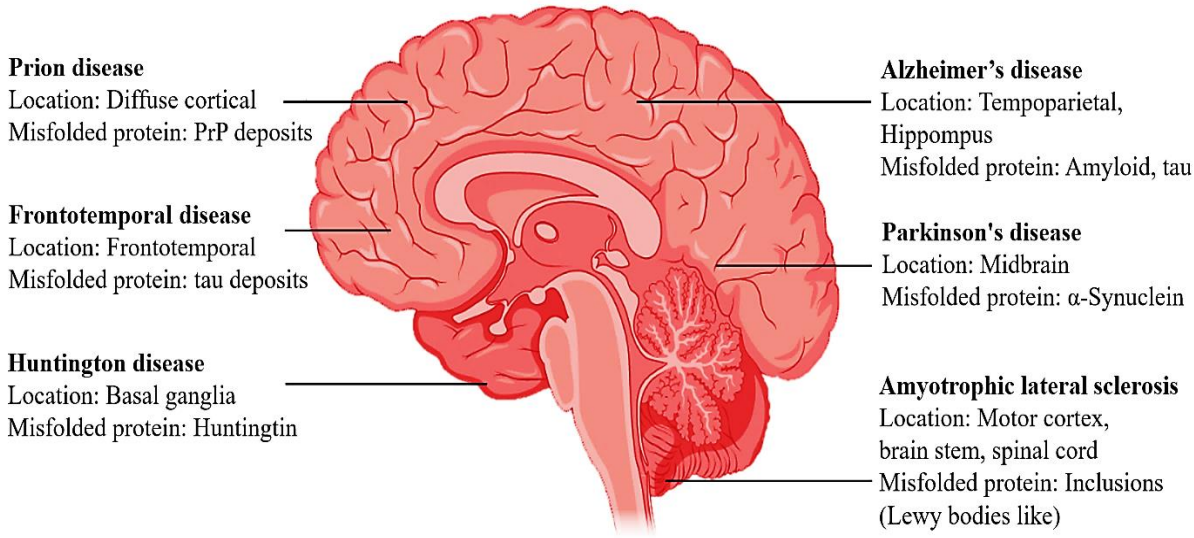


Figure 1.1: The association of different neurodegenerative disorders with particular misfolded proteins in specific brain areas.

ROS, such OH- and ONOO-, are extremely reactive chemicals that may cause oxidative damage to a wide range of natural elements, notably lipids and proteins. They form as remnants of normal metabolic processes in cells as well as in response to exogenous factors such as radiation, toxins, and inflammation. ROS have the ability to oxidise and alter lipids, resulting in LPO. The destructive effect of ROS on unsaturated fatty acids in the membranes of cells leads to the formation of hydroperoxides of lipids and other reactive lipid species (Teleanu et al., 2022). Lipid peroxidation can compromise membrane integrity and generate more ROS, spreading oxidative damage. ROS can also cause oxidative changes to proteins (Feitosa et al., 2018). ROS have the ability to directly oxidise the residues of amino acid inside proteins, leads in the creation of protein carbonyls, disulphide linkages, and other changes (Carvalho et al., 2017). These alterations can change the structure and function of proteins, affecting their normal cellular activity. In terms of nucleic acids, the heterocyclic bases of RNA and DNA, such as guanine, are particularly more prone to oxidative damage induced by ROS. Guanine is more sensitive since it has a lower oxidation potential than other bases. When ROS attack guanine, 8-hydroxy-2-deoxyguanosine is produced in DNA and 8-hydroxyguanosine (8-OHG)

is produced in both DNA and RNA. If not repaired appropriately, these oxidative DNA/RNA lesions can disrupt normal base pairing and result in mutations, breakage of DNA strand, and genomic instability (Radi et al., 2014).

The principal generator of ROS within cells is mitochondria. Oxidative stress can impair mitochondrial function, leading to further ROS generation and an ongoing process of oxidative damage (Schieber et al., 2014). Oxidative damage can trigger an inflammatory cascade response in brain (Valko et al., 2007). Chronic inflammation attributes to neurodegeneration by promoting the release of inflammatory molecules and activating immune cells that can damage neurons (Radak et al., 2011). Misfolding of protein and aggregation may trigger by oxidative damage, such as α-Syn in PD or tau and β-amyloid in AD. Such aggregates of proteins are hazardous to neurons and contribute to disease progression as depicted in **Figure 1.2**.

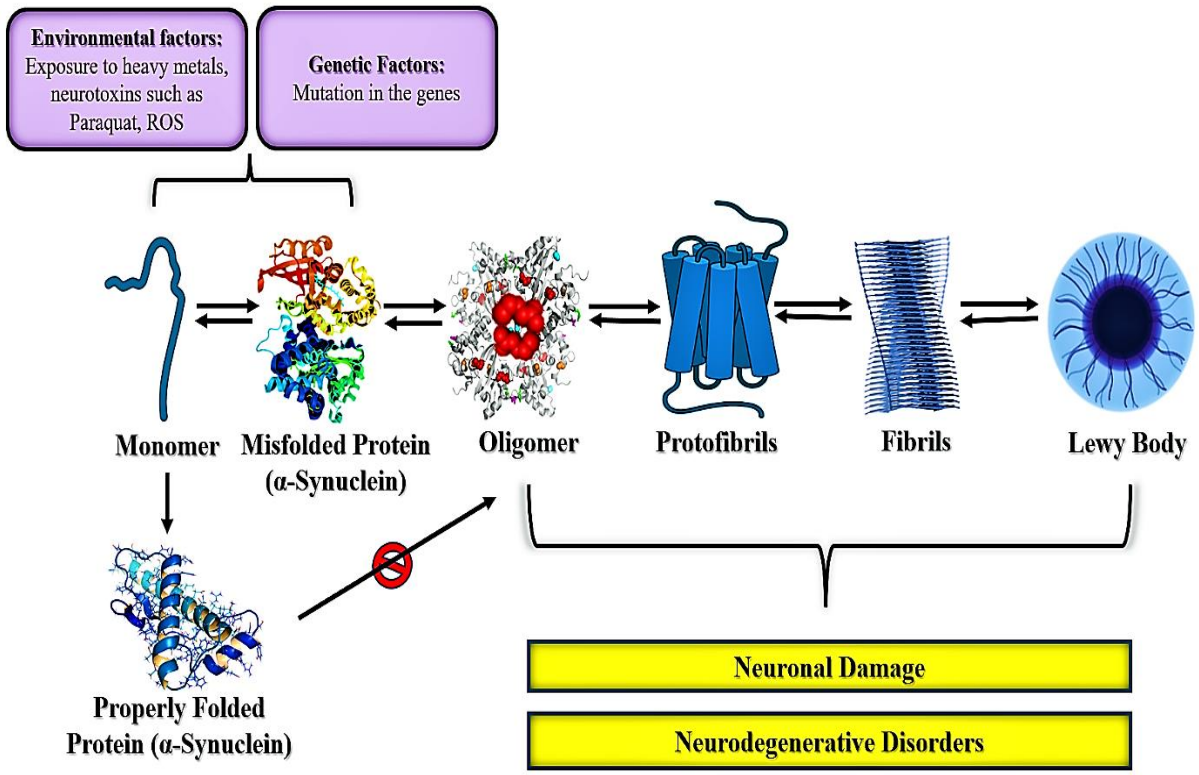


Figure 1.2: The mechanism of large aggregation development in neurodegeneration: Under normal circumstances, α-Syn is a soluble protein that affects neurotransmitter release and synaptic activity. However, in certain cases, genetic abnormalities as well as environmental influences can cause alpha-synuclein to misfold into an aberrant shape. Misfolded alpha-synuclein has a propensity to aggregate, which means that several individual protein molecules join together to create bigger clumps or aggregates. These aggregates can take the shape of soluble oligomers, protofibrils, or insoluble fibrils. Fibrillar aggregates are the primary elements of Lewy bodies, which are pathological signature formations detected in brains of people with PD.

In neurological conditions, Antioxidant enzyme activity, such as glutathione peroxidase and catalase is normally reduced. This reduction in antioxidant capability aggravates oxidative damage. The course of neurodegenerative diseases such as PD, HD, ALS, and AD are influenced by oxidative damage (Ischiropoulos and Beckman, 2003). Because of its high oxygen demand, substantial lipid content, and weak antioxidant defences, the brain is extremely vulnerable to oxidative stress. In neurodegenerative illnesses, oxidative damage can have a number of negative consequences (Juan et al., 2021).

Protein misfolding and aggregation are closely associated with oxidative damage in the context of neurodegeneration. ROS are formed as a result of stress from oxidation and have the ability to directly change proteins. ROS can oxidize amino acid residues within proteins, such as methionine, cysteine, and tyrosine, altering their chemical properties (Li and Borchardt,1995). These oxidative modifications can disrupt protein folding and stability, increasing the likelihood of protein misfolding. Oxidative stress can disrupt the cellular machinery responsible for maintaining protein homeostasis, which includes protein folding, quality control, and degradation systems (Moreau and King, 2012). Molecular chaperones, which assist in proper protein folding, can be impaired by oxidative damage, compromising their ability to prevent misfolding. Additionally, oxidative stress can disrupt the function of ubiquitin system and autophagy, that are in charge of removing misfolded proteins. Free radicals, which are extremely reactive chemicals, attack and destroy lipids (fats) in cell membranes, resulting in lipid peroxidation (Yin, 2011). It is one of the fundamental processes behind oxidative damage, that is described as an imbalance among the body's antioxidant responses and free radical formation. Diverse clinical problems, including neurologic ailments, have been associated to LPO and oxidative damage. In neurodegenerative illnesses such as AD, ALS, PD, and HD, oxidative stress exerts a crucial role in disease progression. Mitochondria are essential cellular organelles involved in energy production, calcium homeostasis, and various other cellular processes. They are critical for neuronal function and are especially sensitive to oxidative damage, which has been analogous to neurodegenerative illnesses. Oxidative stress-induced dysfunction of mitochondria is considered a main mechanism in the pathogenesis of neurological pathologies (Gaki and Papavassiliou, 2014).

Inflammation with oxidative stress are two factors that work together to cause neurodegeneration (Gkekas et al., 2021). Oxidative damage, referred to as an imbalance among ROS generation and intracellular antioxidant defence systems, can produce inflammatory responses in the brain, exacerbating processes that lead to dementia. There are several cascades

that contribute to the processes through which oxidative damage produces inflammation in degeneration. Oxidative stress is implicated in neuronal cell death found in neurodegenerative diseases. Pathways of processes are one manner by which oxidative damage leads to neurodegenerative cell death (Crispino et al., 2020). Through the oxidation of nucleic acids, oxidative stress may induce damage to mitochondrial and nuclear DNA. DNA damage accumulation can impede DNA repair systems and contribute to genomic destabilisation. Unrepaired or incorrectly repaired DNA can activate cell death pathways. Oxidative stress may result in degradation and protein misfolding inside neurons. Proteins that have been oxidised or misfolded can form aggregates and compromise cellular function. Protein clumps such as amyloid-beta and tau build in AD and can activate neuronal cell death mechanisms (Abramov et al., 2020).

Rotenone is an organic substance found in the roots of plants such as the jicama vine and the barbasco plant. It has been used for various purposes throughout history, including as a natural insecticide and piscicide (a substance used to kill fish). It has also been employed in research to study the mechanisms of neurodegeneration, particularly in relation to PD (Siddiqui et al., 2013).

Systemic administration of medicines to the CNS is a substantial obstacle, owing to their short half-life, considerable first-pass metabolism, low access to the brain, and potential adverse effects when reaching non-target peripheral organs (Begley, 2004). As a result, developing systemic delivery mechanisms with higher potency is critical for CNS pharmacotherapy. Cholinesterase (ChE) inhibitors, tacrine, N-methyl-D-aspartate (NMDA) agonists in connection with Vitamin D, levodopa, or other dopaminergic agonists and memantine have never been utilised in conventional medicine or therapeutics to alleviate anything apart from motor symptoms by replenishing neurotransmitters (Camberos and Massieu, 2020). However, prolonged use of these medications can have substantial side effects, including other motor challenges (Shabir and Singh, 2022). To stop or reduce the progression of numerous neurodegenerative disorders, researchers must create new natural neuroprotective agents due to the inadequacy of treatment medicines (Rahman et al., 2021). By activating the Akt/Nrf2/PI3K pathway and eliminating free radicals, nutraceuticals and other phytonutrients have been proven to have protective benefits and to reduce the consequences of neurodegenerative conditions as illustrated in **Figure 1.3** (Qu et al., 2020).

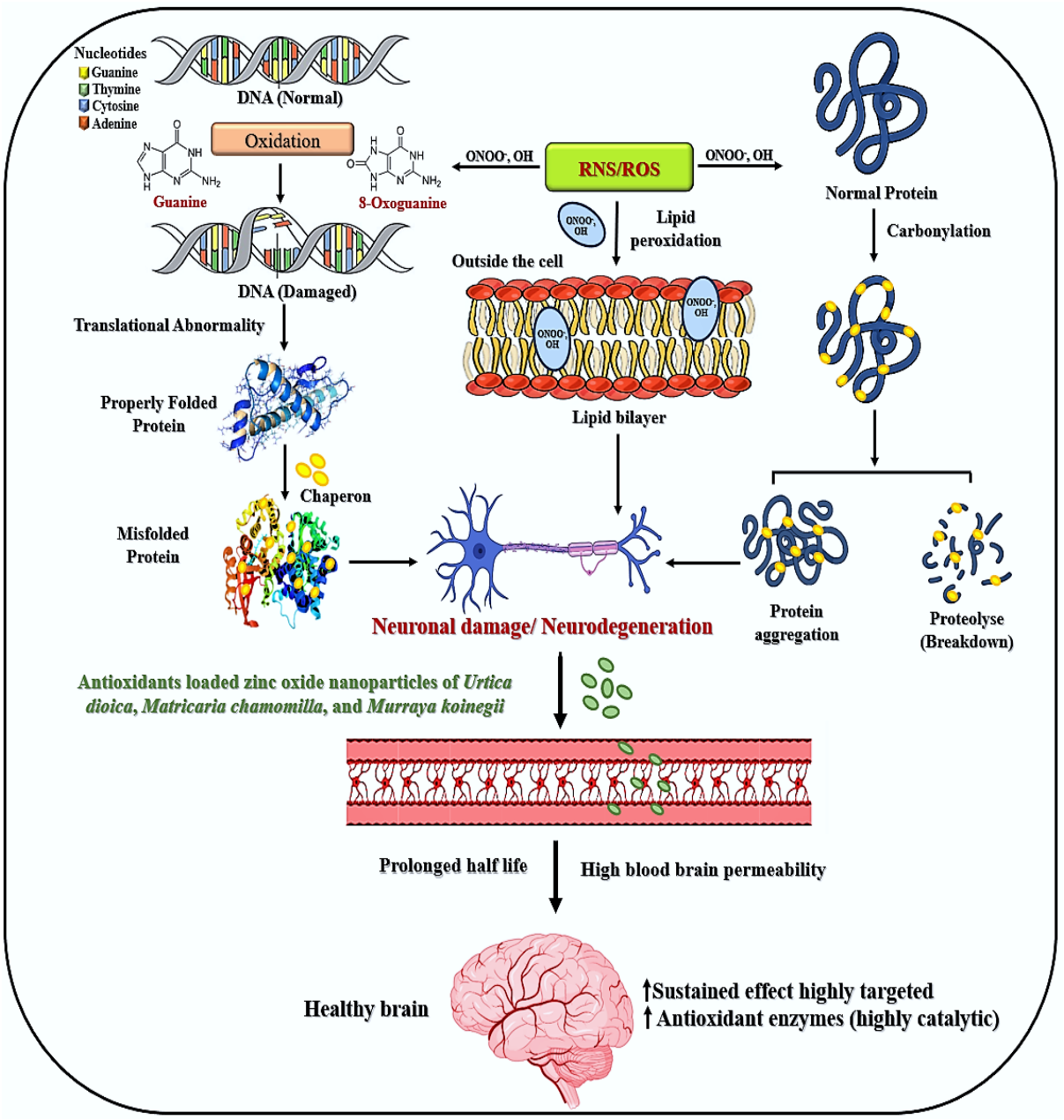


Figure 1.3: Oxidative stress: A key modulator in neurodegeneration. Reactive oxygen species can attack DNA bases, leading to the formation of modified bases like 8-oxoguanine (8-oxoG) and 8-hydroxyguanine (8-OHG). These modifications can impair DNA replication and transcription, potentially leading to mutations and genomic instability. Neuronal membranes have been associated to be enriched in PUFAs; which are especially reactive to ROS and leads to neurodegeneration.

The WHO (World Health Organization) estimates that 78% of people worldwide currently use phytomedicine as their primary source of treatment (Nath and Debnath, 2022). In this regard, researchers investigated *Urtica dioica* (UD), often known as stinging nettle, an evergreen edible plant in the *Urticaceae* family. The therapeutic properties of medicinal plants are determined by the concentration of bioactive substances in nettle plants such as tannins,

carotenoids, vioxanthin, terpenoids, alkaloids, glycosides, lycopene, essential oils, saponins, and protein components, among other ingredients. Stinging nettle plant contains phenolic elements including rutin, chlorogenic acid, p-coumaroyl malic acid, isorhamnetin-3-O-rutinoside, 2-O-caffeoyl malic acid, isoquercetin, isorhamnetinhexoside, and kaempferol 3-O-rutinoside . This plant also contains a number of fatty acids, including linolenic acid, palmitic acid, phenolic compounds, and certain vital amino acids (Anand et al., 2022). Stinging nettle contains antiinflammatory qualities and has been used traditionally to alleviate symptoms of inflammatory conditions such as arthritis and allergies. It may inhibit inflammatory mediators and reduce pro-inflammatory cytokine production. Antioxidants found in UD may scavenge free radicals and minimise oxidative stress. This property is important in protecting cells and tissues from damage caused by oxidative stress. Some studies suggest that UD may have analgesic effects. It may help alleviate pain associated with conditions such as arthritis, joint pain, and muscle pain (Yousuf et al., 2022). It has been traditionally used as a diuretic, promoting increased urine production. It may help with conditions like edema and urinary tract infections. Stinging nettle has been used to alleviate symptoms of allergies, such as hay fever. It may help by suppressing the release of inflammatory mediators involved in allergic reactions. UD exhibits antimicrobial properties against various pathogens, including bacteria and fungi. It may play a function in the treatment of some infections. Some research suggests that *U. dioica* may have anti-diabetic properties. It may aid in the regulation of blood sugar levels and the improvement of insulin sensitivity. The plant has traditionally been used topically for wound healing. It may promote tissue repair and aid in wound closure (Kregiel et al., 2018).

Matricaria chamomilla (MC), sometimes known as chamomile, is a medicinal plant in the Asteraceae family that has been acclaimed as a therapeutic star. Major bioactive components are terpenoids, apigenin-7-O-glucoside, apigenin, chlorogenic acid, caffeic acid, flavonoids (apigenin, quercetin, luteolin, and patuletin), luteolin, glycosides, luteolin-7-O-glucoside, coumarins (herniarin and umbelliferone), hydroxycoumarins, α-bisabolol, and azulenesse or chamazulene (Purseglove, 1989). Chamomile flower extracts have been shown to exhibit antioxidant and free radical scavenging properties. MC contains compounds, such as chamazulene and flavonoids, that exhibit anti-inflammatory capabilities. It may assist in reduction of inflammation in various conditions, including skin irritations, gastrointestinal disorders, and inflammatory diseases. Chamomile has been traditionally used to promote relaxation and relieve anxiety (Baser et al., 2006). It may have mild sedative properties, aiding in sleep and promoting a sense of calmness. Antioxidants found in MC extract could help in

the neutralization of free radicals and protection of cells from oxidative stress. This property may contribute to its potential benefits in promoting overall wellness and decreasing the probability of chronic diseases. Chamomile has been used to alleviate digestive discomfort, including indigestion, bloating, and stomach cramps. It may help relax the smooth muscles of the digestive tract and exhibit antispasmodic effects. MC extracts have shown antibacterial activity against numerous pathogens, including fungi and bacteria (Jabri et al., 2017). It may help in growth inhibition of certain microorganisms and contribute to the treatment of infections. Topical application of MC has been utilized for its potential benefits in promoting skin health. It may have soothing and anti-inflammatory effects, making it useful in treating skin irritations, eczema, and mild wounds. Some studies suggest that chamomile may have anti-diabetic properties by helping regulate blood sugar levels. It may improve insulin sensitivity and glucose metabolism. In preclinical investigations, MC extracts demonstrated promising benefits in suppressing cancer cell proliferation and causing apoptosis (also called programmed cell death). However, more research must occur to establish its worth in cancer treatment (Miraj and Alesaeidi, 2016).

Murraya koenigii (MK) is a Rutaceae plant that is often known as curry leaves and is used in cooking. So far, the active components found from the leaves include D-α-terpinol, dipentene, D-sabinene, koenigine-quinone A, caryophyllene, and koenigine-quinone in MK essential oil. MK leaves include phytoconstituents such as proteins, cellulose, oxalic acid, minerals, carotene, calcium, nicotinic acid, vitamin A, carbohydrates, and vitamin C (Ningappa and Srinivas, 2008). MK extract also contains O-methyl mahanine, O-methyl murrayanne, carbazole alkaloids, koenigine, koenine, koenimbine, crystalline glycosides, iso-mahanimbin, and koenidine. These three herbs include phytochemicals with proven protective properties, including quercetin, alkaloids, reducing sugars, vitamins, and polyphenols. Curry leaf contains various antioxidants, such as phenolic compounds and flavonoids, that can eliminate free radicals and decrease oxidative stress. This characteristic may contribute to its potential advantages in reducing the probability of chronic illnesses while avoiding oxidative damage (Selvan *et al*., 2022). Compounds present in curry leaf, including carbazole alkaloids, have been found to exhibit anti-inflammatory properties. It may help reduce inflammation and alleviate symptoms associated with inflammatory conditions. MK has historically been utilised in the practise of Ayurveda for its anti-diabetic effects. It may help to regulate glucose levels, improve insulin sensitivity, and reduce diabetes complications. Curry leaf extracts exhibit antibacterial properties against various ailments, including mould, bacteria, and viruses. It may

have the potential in growth inhibition of microorganisms and contribute to the management of infections. In traditional medicine, curry leaf has been used to promote digestion and alleviate gastrointestinal issues. It may help stimulate digestive enzymes, improve gut motility, and relieve indigestion and nausea (Kusuma et al., 2011).

Studies have suggested that MK possesses hepatoprotective effects, meaning it can protect the liver from damage caused by toxins or certain diseases. It may help improve liver function and prevent liver damage. Some research indicates that curry leaf may have neuroprotective properties. It may help protect against neurodegenerative diseases and cognitive decline by lowering inflammation and oxidative stress in the brain. Certain compounds found in MK have demonstrated potential anti-cancer effects in preclinical studies. They may decrease cancer cell proliferation and trigger apoptosis (Bhandari, 2012).

Delivering medicines and nutritional antioxidants to particular brain sites is complicated by the presence of the blood-cerebrospinal fluid barrier (BCFB), blood-brain barrier (BBB), and other physiological variables (Campos et al., 2014). Despite the screening of several pharmacological candidates, only a few options, such as tacrine, memantine (NMDA inhibitor), donepezil, galantamine (AChE and BChE inhibitors), rivastigmine, and curcumin are now used in the therapeutic treatment of NDs. The CNS is the most complex and intricate system in the human body. Because of the CNS's complicated nature, there are several restrictions to focused medication delivery from the blood brain to the CNS. These include a lack of understanding of brain cell accessibility, pharmacokinetics, or pharmacological activity, as well as undesirable effects and unanticipated interactions of off-targets-drugs with unspecific receptors and enzymes (Li et al., 2021). This is due to the highly complex pharmacy of various medications, the long-time period of neurological ailments, medication inefficiency after progression of disease, incorrect dosing, the patient population's genotype and different responses to medications, and the volatility index of assessed medications. Additionally, the brain's abnormalities will only intensify (Ding et al., 2020). Novel approaches for the improved crossing of the BBB must be developed in order to progress in the effective therapies (Swierczewska et al., 2018). The implementation of novel nanotechnology-based technologies, such as nanoparticles as nanocarriers, may allow drugs/medicine to cross the BBB and reach the target brain area (Patra et al., 2018).

Nanotechnology is a novel and fast evolving technique with several potential applications. Nanoparticles (NPs) are tiny particles typically ranging from 1 to 100 nanometres. They are

categorised as zero-dimensional (quantum dots and nanoparticles), one-dimensional (nanofibers, nanotubes, and nanowires), and two-dimensional (graphene). In recent years, nanomaterials such as nanotubes, nanoparticles, nanofibers, and quantum dots have found widespread use in biomedical fields such as delivery of drugs, biological imaging and biosensors. NPs can be employed to encapsulate and distribute therapeutic medications to particular target areas in the body, increasing medicinal efficacy while decreasing adverse effects (Bhattacharya et al., 2022). Nanoparticles can be engineered to carry therapeutic agents, such as drugs or biomolecules, to specific locations in the brain. These nanoparticles can cross the BBB, a protective barrier that prevents many substances from entering the brain, and deliver the drugs directly to the affected areas. NPs can be designed to provide neuroprotective effects. Additionally, nanoparticles can be used to deliver growth factors or other molecules that promote neuronal survival and regeneration, potentially slowing down disease progression (Hernandez and Shukla, 2022).

Green synthesis of NPs refers to the production of nanoparticles using ecologically friendly and sustainable technologies (Letchumanan et al., 2021). These methods aim to eliminate or reduce the use of toxic chemicals and poisonous procedures typically associated with conventional nanoparticle synthesis. Green synthesis offers several advantages, including lower environmental impact, cost-effectiveness, and potential biocompatibility for biomedical applications (Nasrollahzadeh et al., 2019). Plant fractions, microorganisms (fungi and algae), bio-waste Materials (agricultural waste and other bio-waste materials), sun irradiation, and green solvents are some typical ways for green nanoparticle production (Singh et al., 2018). Plant extracts, such as leaves, roots, stems, or fruits, are widely employed in green synthesis. These extracts contain natural chemicals such as flavonoids, polyphenols, terpenoids, and proteins, which can function as stabilising agents in the creation of NPs (Abomuti et al., 2021). The fraction is mixed with metal precursors, and the reduction reaction occurs under mild conditions, leading to the formation of nanoparticles. Various plants, such as *Aloe barbadensis* miller, *Camellia sinensis*, *Azadirachta indica*, and *Curcuma longa*, have been used for nanoparticle synthesis (Ahmed et al., 2017).

Using plant extracts from various plant species, many metals, including silver (Ag), zinc oxide (ZnO), gold (Au) and many others, have been employed in the phyto-fabrication of NPs (Singh et al., 2018). Zinc oxide high pharmacological qualities have enhanced its usage in healthcare applications such as antioxidant, antibacterial, and anticancer (Mishra et al., 2017). Furthermore, zinc oxide, alongside four other zinc compounds, has been designated as a

generally recognised as safe (GRAS) chemical by the US-FDA (Food and Drug Administration). Zinc oxide nanoparticles are nano-sized particles composed of zinc and oxygen atoms. They possess unique chemical, physical, and optical characteristics (Prasad and Lall, 2022). Because of their diverse features and possible uses, ZnO-NPs have received a lot of interest in a variety of sectors. Even though UD (Singh et al., 2022), MC (Ogunyemi et al., 2019), and MK (Philip et al., 2011) NPs had been employed in several *in vitro* and *in vivo* research, there is currently less information available regarding the beneficial impact of these NPs on cellular and neurological issues.

The mechanism of plant-derived nanoparticles (NPs) for the therapy of neurodegenerative disorders involves several pathways and interactions within the CNS. Plant-derived NPs, such as ZnO NPs, often possess inherent antioxidant properties due to the presence of phytochemicals. These nanoparticles can scavenge free radicals and ROS in the brain, lowering oxidative damage, which has been linked to neurodegeneration. By protecting neurons from oxidative damage, plant-derived NPs may delay or halt the progression of neurological diseases (Babazadeh et al., 2020). Phyto-engineered NPs may exert neuroprotective effects by enhancing neuronal survival and promoting neuronal growth and differentiation. These NPs have the ability to activate neurotrophic factors including BDNF, which are necessary for neuronal growth and maintenance. Green NPs can also influence signalling mechanisms involved in neuroprotection and cell survival, such as the Akt/PI3K and MAPK/ERK pathways. Some neurodegenerative illnesses, such as AD, are characterised by an abnormal buildup of metal ions (copper, zinc) and protein clumps (amyloid-beta) in the brain. Plant-derived NPs, including ZnO-NPs, can act as metal chelators, binding to metal ions and preventing their harmful interactions. Additionally, these NPs may inhibit the aggregation of amyloid-β protein, reducing the generation of toxic amyloid plaques associated with neurodegeneration (Squillaro et al., 2018).

Plant-based nanoparticles can be anti-inflammatory by suppressing pro-inflammatory mediators including cytokines and enzymes like iNOS and COX-2. They may modulate the immune response and reduce inflammation in the brain. They can enhance neurotrophic factors including NGF and BDNF, which support neuronal health and function. Additionally, these nanoparticles may stimulate neurogenesis (generation of new neurons) and synaptogenesis (formation of new synapses), which are vital for neuronal repair and recovery. Metal ion abnormal buildup, particularly copper and zinc, is seen in neurological conditions and leads to neuronal damage (Geetha and Ramachandran, 2021).

Certain plant-based nanoparticles have metal chelating properties, which means they can bind to these metal ions and help maintain metal ion homeostasis in the brain. Plant-synthesized NPs can be engineered to possess properties that facilitate their transport across the BBB (Zafar et al., 2020). Once in the brain, these NPs can deliver therapeutic agents, such as antioxidants, anti-inflammatory compounds, or neuroprotective drugs, directly to the affected regions. The targeted delivery of drugs using green NPs may enhance their effectiveness while minimizing systemic side effects (Prasanna and Upadhyay, 2021).

Drosophila melanogaster, commonly referred to fruit fly, is a tiny insect in the *Drosophilidae* family. It is one of the most intensively researched organisms in biological study, notably in developmental biology and genetics. Adult *Drosophila* flies are about 3-4 mm long and have a tan or light brown body with red eyes (Hodgetts, 1973). *Drosophila* has a relatively small genome size of approximately 180 million base pairs, with around 14,000 protein-coding genes. The fly's genetic makeup shares many similarities with that of humans, making it a useful model organism for understanding human genetics and disease. However, its small size, short lifespan, and lack of complex emotions and cognitive abilities make it a less controversial choice compared to using higher organisms in scientific studies (Aryal and Lee, 2018).

In this regard, this study used the green method to fabricate ZnO-NPs from the UD, MC, and MK fractions. The phyto fabricated ZnO-NPs were analyzed utilizing SEM, FT-IR, UV-vis, Zeta potential, XRD technique, EDX analysis, and DLS method. The well diffusion approach was utilized to evaluate the antibacterial abilities of biosynthesized ZnO-NPs against *Staphylococcus aureus* and *Escherichia coli*. Moreover, we aimed to explore the therapeutic potential of phytofabricated ZnO nanoparticles against a commonly used neurotoxic organic pesticide rotenone *in vivo* in *Drosophila* (Oregon R^{+}) at cellular, neurochemical, biochemical, and molecular level for the first time. We also evaluated its behavioral parameters on *Drosophila*, which has previously been proved to be a valuable indicator for neurodegenerative diseases.

CHAPTER-2

REVIEW OF LITERATURE

2.1 PREVALENCE AND INCIDENCE OF NEURODEGENERATIVE DISEASES

Neurodegenerative disorders are the leading factor in disability and the second main cause of mortality worldwide (Rahman et al., 2023). The report from the Global Burden of Diseases (GBD) 2019 provided the most comprehensive and up-to-date estimates of the national, regional, and global burden from neurodegenerative disorders. Particularly in middle- and low-income countries, neurodegenerative disease-related disability and mortality have increased significantly during the past 30 years (Feigin et al., 2022). Further increases are anticipated globally due to population growth and ageing. It is predicted that 55 million individuals globally had dementia in 2019, and that this figure will rise to 139 million by 2050 (GBD, 2022). The UN General Assembly report, emphasized that progress in eliminating the prevalence of noncommunicable disorders, particularly neurodegenerative disorders, has been insufficient to reach the UN sustainable development goal by 2030 (WHO, 2021).

The globally estimated prevalence rate of neurodegenerative diseases cases were highest for Alzheimer's disease (62%) followed by Parkinson's disease (17%), Mixed dementia (10%), Lewy body dementia (4%), Fronto-temporal dementia (2%), Vascular dementia (2%), and other neurodegenerative diseases (3%), respectively for 2019 as illustrated in **Figure 2.1** (GBD, 2022).

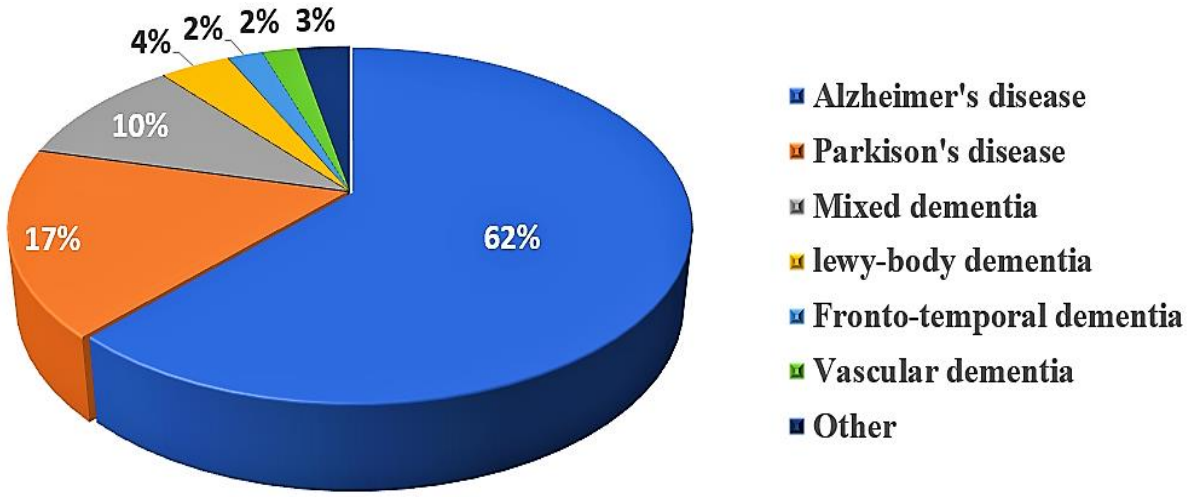


Figure 2.1: The global prevalence rate of individual neurodegenerative disorder as percentage of total neurodegenerative disorders: the Global Burden of Disease (GBD) Study 1990-2019 (GBD, 2022).

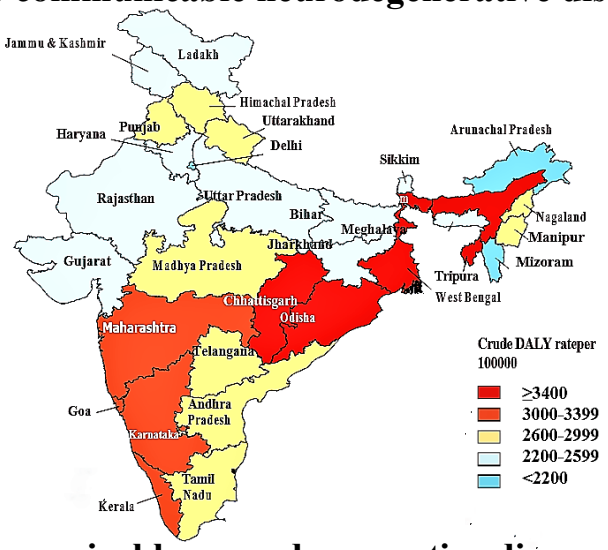
The evolution of neuroepidemiology in India over the last four decades has been traced, with historical milestones highlighted. In India, where epidemiological and demographic shifts are

occurring rapidly, it is also anticipated that the burden of neurological diseases will escalate. The Indian population accounts for around 18% of the global population, with several of its states having numbers comparable to that of entire countries (Zhong and Zhu, 2022). The prevalence rates of the broad range of brain diseases in different parts of the country ranged from 967-4070, with a mean of 2394 per 100,000 population, implying that over 30 million individuals experienced neurological disorders (Ayeni et al., 2022).

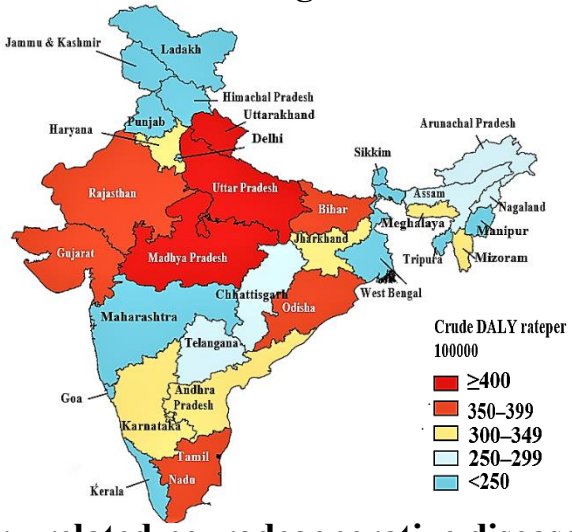
The first comprehensive analyses of the disease burden caused by neurodegenerative diseases and their patterns in all states of India were published in The Lancet Global Health by the India State-Level disease Burden Initiative. These neurodegenerative diseases include noncommunicable neurological ailments (stroke, PD, epilepsy, AD, headache disorders, cerebral palsy, motor neuron diseases, Huntington's disease, multiple sclerosis, central nervous system (CNS) cancer, and other neurodegenerative diseases), communicable neurodegenerative diseases (meningitis, tetanus, and encephalitis), and injury related neurodegenerative diseases (spinal cord injuries and traumatic brain injuries) (Choudhary et al., 2021). The proportion of noncommunicable neurodegenerative conditions to total disability-adjusted life years (DALYs) from all causes in India more than doubled from 4.0% in 1990 to 8.2% in 2019, whereas communicable neurodegenerative disorders declined from 4.1% in 1990 to 1.1% in 2019. Injury-related neurodegenerative diseases accounted for 0.6% of total DALYs in 2019, up from 0.2% in 1990. In India, the fraction of total DALYs owing to all neurological conditions (including communicable, noncommunicable, and injury-related disorders) increased marginally from 8.3% in 1990 to 9.9% in 2019 as depicted in **Figure 2.2** (GBD, 2021).

In 2019, an estimated 369 million individuals in India had Alzheimer's disease and 128900 people died as a result of these disorders. The crude DALY rate of Alzheimer's disease varied by 3.3 times amongst states with the highest rates in Himachal Pradesh, Kerala, Andhra Pradesh, and Goa (Dhiman et al., 2021). In case of Parkinson's disease, it was estimated that 771000 people were suffering from this condition and 45300 people had died. There was 2.3 times difference in the crude DALY rate of Parkinson's disease amongst the states, with Goa having the highest rate. Noncommunicable neurodegenerative diseases were most prevalent in all other age groups, whereas communicable neurodegenerative diseases accounted for a majority of overall neurodegenerative diseases burden among children under the age of five (Garg, 2021).

(A) Non-communicable neurodegenerative diseases



(B) Communicable neurodegenerative diseases



(C) Injury-related neurodegenerative diseases

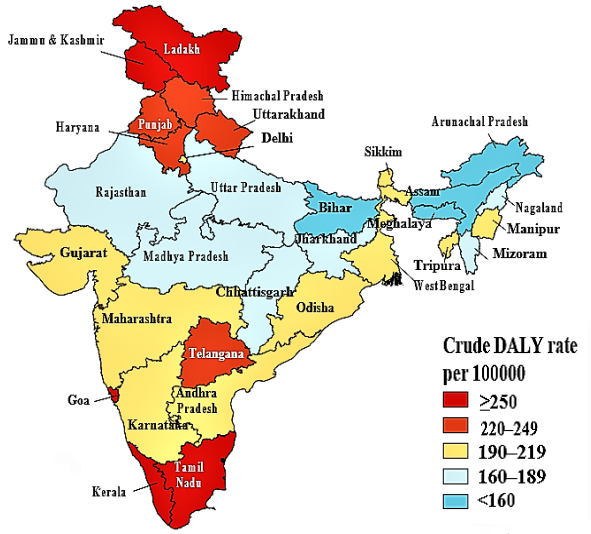


Figure 2.2: The rising incidence of disability-adjusted life years (DALYs) of non-communicable, communicable, and injury related neurodegenerative diseases across the states of India: the Global Burden of Disease (GBD) Study 1990-2019 (GBD, 2021).

2.2 NEURODEGENERATIVE DISORDERS

A neurodegenerative disease is defined by the gradual impairment of neuron function or structure, an occurrence known as neurodegeneration (Calabresi et al., 2023). Many are inherited, while others are the consequence of toxic or metabolic processes, and yet others are the outcome of infections. Neurodegenerative disorders impose a tremendous social, medical, and economical burden on society due to their prevalence, morbidity, and death. Neurodegenerative disorders include Amyotrophic lateral sclerosis, Parkinson's disease, Multiple sclerosis, Alzheimer's disease, Prion diseases, and Huntington's disease. Neurodegeneration can be identified at a variety of levels in the brain's neural circuitry, ranging from molecular to systemic (Wilson et al., 2023). These conditions are considered incurable since there is no known way to stop the slow deterioration of neurons; nonetheless, research has revealed that inflammation and oxidative damage are the two key contributing factors to neurodegeneration (Golpich et al., 2017). Biomedical research has discovered several correlations between these disorders at the subcellular level, such as aberrant protein assemblies including proteinopathies and triggered cell death (Jellinger, 2010). These connections imply that treatment achievements against one neurodegenerative condition may benefit other pathologies as well (Federico et al., 2012).

2.2.1 Classification of Neurodegenerative diseases

Neurodegenerative conditions are an assortment of diseases that are characterised by the reluctant degeneration and decline in the structure or function of neurons (nerve cells) in the brain's peripheral nervous system (Fumia et al., 2022). These conditions typically affect the CNS brain and spinal cord and frequently result in cognitive loss, movement disorders, and other neurological symptoms (Tesco and Lomoio, 2022).

Here are some common neurodegenerative disorders:

a) Alzheimer's disease (AD)

AD is the most prevalent form of cognitive impairment, affecting 45 million people worldwide. It is a degenerative neurological ailment that damages the brain (Guo et al., 2022). It typically affects older adults, although there are cases of early-onset Alzheimer's that can occur in individuals under the age of 65. The specific etiology of AD is unknown; however it is thought to be a mix of hereditary, environmental factors, and lifestyle (Nandi et al., 2022). AD is distinguished by the accumulation of abnormal protein deposits in the brain known as tau

tangles and Aβ plaques. Plaques are made up of small peptides called amyloid beta (Aβ) that are 39-43 amino acids long. Amyloid beta is an element of a larger protein called amyloid precursor protein (APP), which is a protein with a transmembrane structure that enters the membrane of neurons. APP appears to be involved in normal neuron development, survival, and repair after damage (Bai et al., 2022). Enzymes such as γ-secretase and β-secretase break APP into smaller pieces. One of these pieces produces Aβ fibrils, which can self-assemble into thick extracellular amyloid plaques. These deposits disrupt the communication between brain cells, leading to the progressive deterioration of cognitive functions such as memory, thinking, and behavior as depicted in **Figure 2.3** (Kinney et al., 2018).

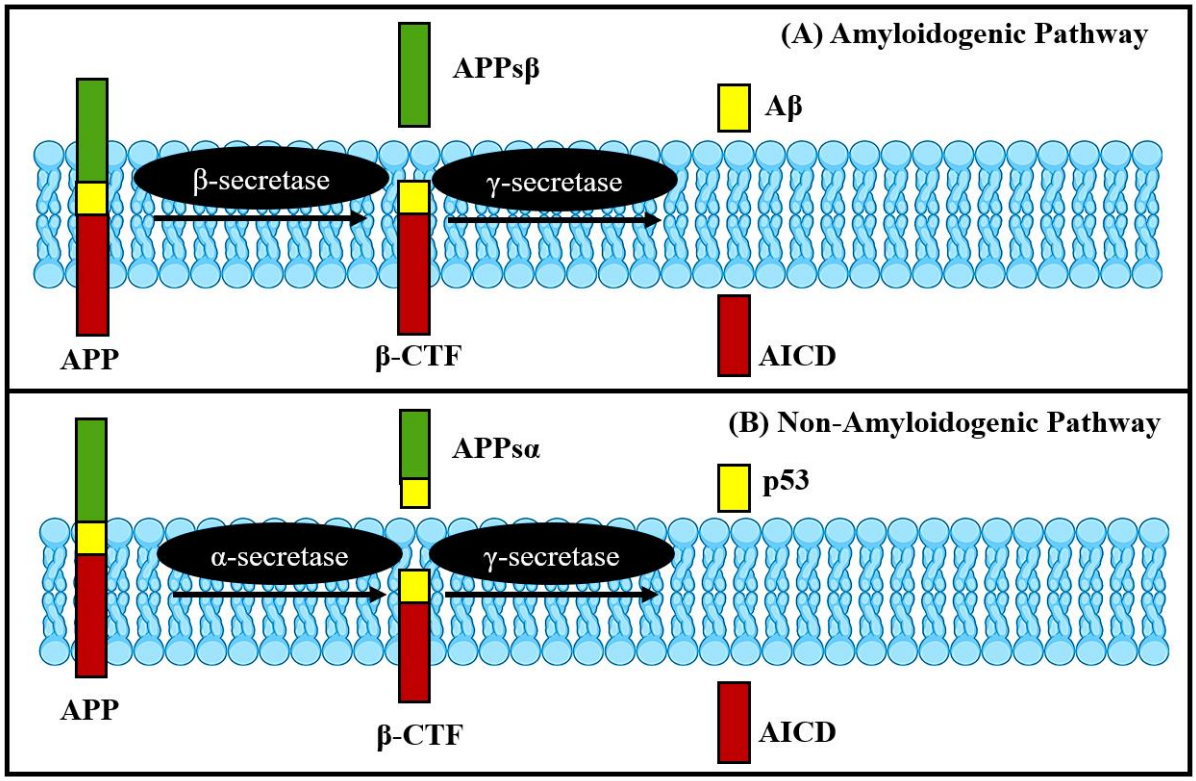


Figure 2.3: Processing of APP: (i) In amyloidogenic pathway, the transmembrane protein secretase act on Amyloid Precursor Protein (APP) and cleave to release APPsβ and β-CTF (C-terminal fragment). The latter is then cleaved by γ-Secretase and produce P3 and AICD (amyloid precursor protein intracellular domain). (ii) In non-amyloidogenic pathway, the transmembrane protein α-Secretase act on Amyloid Precursor Protein (APP) and cleave to release APPsα and α-CTF (C-terminal fragment). The latter is then cleaved by γ-Secretase and produce P3 and AICD (amyloid precursor protein intracellular domain)

Aβ is usually found in two forms: Aβ40 and Aβ42. The amyloidogenic pathway produces Aβ from Amyloid Precursor Protein (APP), a membrane-bound protein, by the activity of β and γ-secretases (Gallardo and Holtzman, 2019). In amyloidogenesis, APP is cleaved by β-secretase, which generates APPsβ and a β-C-terminal fragment, and then γ-secretase, which

generates Aβ, and amyloid precursor protein intracellular domain (AICD), which acts on the transmembrane domain (Ashrafian et al., 2021). However, in the non-amyloidogenic route, α-secretase cleaves APP inside the Aβ sequence, limiting its synthesis. In brief, α-secretase cleaves the ectodomain of APP, resulting in APP fragment and α-CTF. α-CTF is then degraded into AICD and P3 peptide by γ-secretase (Maurya et al., 2023).

The symptoms of AD can vary from individual to individual, but they generally worsen over time. In the early stages, individuals may experience mild memory loss and difficulty with concentration (Joe and Ringman, 2019). As the disease progresses, they may have trouble with language, recognition of familiar objects and people, and performing everyday tasks. In the later stages, individuals may become disoriented, have significant memory loss, and require assistance with daily activities (Kivipelto et al., 2018). Diagnosing Alzheimer's disease involves a comprehensive evaluation of medical history, physical examination, neurological tests, and assessments of cognitive function. Brain imaging techniques such as positron emission tomography (PET) or magnetic resonance imaging (MRI) scans may also be used to aid in the diagnosis and rule out other possible causes of dementia (Mahaman et al., 2022).

While there is presently no cure for AD, there are treatment alternatives that may assist with altering symptoms and prevent disease progression. To increase cognitive performance and reduce behavioural symptoms, medications such as memantine and cholinesterase inhibitors are routinely administered.

b) Parkinson's disease (PD)

After Alzheimer's, PD is the subsequent most common neurodegenerative disorder in the elderly (James and Georgopoulos, 2022). It is defined by dopaminergic (DA) neuronal dysfunction in the substantia nigra pas compacta (SNc) and a decrease in DA levels in the brain's nigrostriatal DA pathway. The formation of insoluble inclusions in neurons known as Lewy bodies, which are mostly composed of synuclein, is the primary symptom of this illness (Sezgin et al., 2019). Since neurons govern and control the body's voluntary movements, their degeneration results in reduced bradykinesia, motor function, postural instability, rest tremor, and stiffness. Exogenous causes such as inappropriate use of pesticides and herbicides, exposure to carbon monoxide, organic chemicals, carbon disulphide, plant-derived toxins, and viral and bacterial infections are considered to have a substantial role in the development of PD (Aarsland, 2016). It is also thought that ageing has a role in Parkinson's disease, since as we age, normal cellular processes become increasingly vulnerable to failure, culminating in the

degeneration of dopaminergic neurons. Many genes in the Parkinson's disease family suffered mutations. To understand the pathophysiology of Parkinson's disease, it is necessary to unravel the process through which mutation led to nigral neuron loss in context (Zhong et al., 2022).

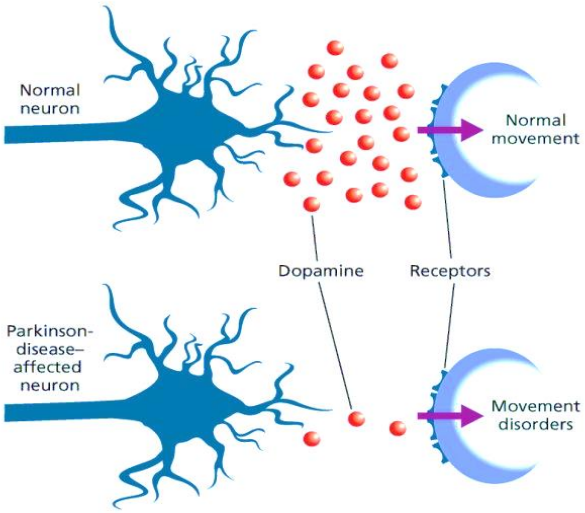


Figure 2.4: Dopamine levels in normal and Parkinson's disease-affected neurons (Tiwari and Pal, 2017).

Tremors (shaking or trembling) in the arms, hands, legs, face, or jaw; bradykinesia (slowness of movement); postural instability (difficulty with coordination and balance); and muscular stiffness or rigidity are the main symptoms of PD as shown in **Figure 2.4** (Hussein et al., 2023). The loss of dopamine-producing cells in the brain causes these symptoms. Dopamine is a neurotransmitter that is required for smooth, coordinated muscle movements (Tolosa et al., 2021). Dopamine deficiency develops when dopamine-producing cells in the brain's substantia nigra die. This imbalance in neurotransmitters causes the motor symptoms associated with PD (Latif et al., 2021).

Sleep disruptions, cognitive abnormalities (such as issues with attention and memory), sensory problems, mood disorders such as anxiety and sadness, and autonomic dysfunction impacting processes such as digestion and blood pressure are all non-motor symptoms of PD (Moustafa et al., 2016). The actual etiology of Parkinson's disease is unknown, however both hereditary and environmental factors are thought to have a role. Some cases of PD are characterised by particular gene alterations, whilst others may be impacted by external variables such as toxicity exposure (Armstrong and Okun, 2020).

There is currently no curative approach for PD, although numerous therapy options are available to control its symptoms. agonists of dopamine, levodopa, and MAO-B inhibitors are medications that can assist boost dopamine levels or imitate its actions in the brain.

c) Huntington's disease (HD)

HD is a hereditary condition that corresponding in the gradual destruction of neurons in the brain (Tabrizi et al., 2022). It is named after George Huntington, the physician who first

described its symptoms in 1872. HD is an inherited disorder, meaning it is passed down from parents to their children through a mutation in the huntingtin gene (Wild and Tabrizi, 2017). The huntingtin gene mutation results in the production of a toxic protein called mutant huntingtin (Kolli et al., 2017). This protein builds up in certain regions of brain, particularly in the cerebral cortex and basal ganglia, resulting in the destruction of brain cells over time. The basal ganglia plays an important role in movement control, while the cerebral cortex is involved in cognition and emotion (Kumar et al., 2015).

The symptoms of HD typically manifest between the ages of 31 and 50, although onset can occur at any age. The most prominent symptoms include movement abnormalities, cognitive decline, and psychiatric disturbances (Dash and Mestre, 2020). These symptoms progressively worsen over time and significantly impact a person's quality of life. Movement abnormalities in Huntington's disease often begin with subtle motor changes, such as involuntary jerking or twitching movements called chorea (Oikemus et al., 2022). As the disease progresses, individuals may experience difficulties with coordination, balance, and voluntary movements. This can lead to problems with walking, speaking, swallowing, and performing daily activities (Aldaz et al., 2019). Cognitive decline in HD affects various cognitive functions, including memory, attention, problem-solving, and executive functions. Individuals may experience difficulties with organizing tasks, making decisions, and planning ahead (Monteys et al., 2017). As the disease advances, severe cognitive impairment can occur, leading to significant challenges in daily living (Weiss et al., 2022). Psychiatric symptoms are also common in HD and can precede the onset of movement and cognitive symptoms. These symptoms may include depression, anxiety, irritability, mood swings, impulsivity, and social withdrawal. Psychiatric disturbances can significantly impact relationships and overall mental well-being (Isaacs et al., 2020).

There is presently no cure for HD, and present therapies try to control symptoms and enhance quality of life.

d) Amyotrophic lateral sclerosis (ALS)

ALS is a neurological condition that primarily affects nerve cells that control voluntary muscles (Feldman et al., 2022). ALS is one of a set of conditions known as motor neuron diseases, which cause the slow deterioration and death of motor neurons in the brain. The initial symptoms of ALS can vary from person to person, but they typically include muscle weakness, muscle twitches (fasciculations), and cramping. As the disease progression starts, individuals

may experience difficulty in walking, speaking, swallowing, and breathing. However, the course and progression of ALS can be unpredictable (Kiernan et al., 2021). Upper motor neurons (UMNs) in the brain are affected by ALS, as are lower motor neurons (LMNs) in the brainstem and spinal cord. The deterioration of these motor neurons affects communication between the brain and the muscles, resulting in muscular atrophy and loss of control (Ashhurst et al., 2022). The exact etiology of ALS is not fully understood. In most cases, the disease appears to occur sporadically without a clear genetic or environmental cause. However, around 5 to10% of cases are inherited, resulting from mutations of genes. Various genetic and environmental factors are being investigated as potential contributors to the development of ALS (Goutman et al., 2022).

ALS is typically diagnosed based on clinical symptoms and ruling out other possible conditions. Physicians may conduct various tests, including electromyography (EMG), nerve conduction studies, muscle biopsies, and imaging tests, to support the diagnosis and rule out other conditions (Yazdani et al., 2022). There is currently no cure for ALS. Treatment focuses mostly on symptom management, enhancing the life quality, and providing supportive care. To delay the course of the condition, medications such as riluzole and edaravone may be administered.

e) ***Multiple sclerosis (MS)***

Multiple sclerosis is an autoimmune disease that damages the brain and spinal cord over time (Kuhlmann et al., 2023). The immune response in MS incorrectly assaults the defensive coating of nerve fibres known as myelin, resulting in inflammation, injury, and scarring (sclerosis) in different locations of the CNS (Kutzelnigg and Lassmann, 2014). This disrupts the natural flow of electrical impulses across the nerves, causing a range of symptoms. MS symptoms differ widely from individual to individual and are determined by the location and extent of nerve damage (Graves et al., 2023). Walking difficulties, weariness, muscular weakness, numbness or tingling in the limbs, eyesight issues, cognitive challenges, bowel or bladder malfunction, and balance and coordination problems are all common symptoms. Symptoms might appear and go or worsen with time (Ghasemi et al., 2017). Primary progressive MS (PPMS), relapsing-remitting MS (RRMS), progressive relapsing MS (PRMS), and secondary progressive MS (SPMS) are all types of MS. The most frequent variety is RRMS, which is distinguished by periods of relapse (symptom worsening) followed by periods

of remission (partial or total healing). PPMS, SPMS, and PRMS involve a more progressive and steady decline in function without distinct relapses and remissions (Wei et al., 2021).

The actual cause of MS is unresolved; however it is likely to be a combination of environmental variables and hereditary. It is thought to occur when a person with a genetic predisposition is exposed to certain environmental triggers, leading to an abnormal immune response against the myelin in the CNS (Wingerchuk and Carter, 2014). Factors such as vitamin D deficiency, smoking, certain infections, and geographical location have been implicated in MS development. MS diagnosis might be difficult because there is no one test that can confirm the condition. To assess the existence of inflammation and injury in the CNS, physicians often use a mix of neurological examination, medical history, imaging tests (such as MRI), and cerebrospinal fluid studies. The diagnosis may require ruling out other conditions with similar symptoms (Brownlee et al., 2017).

There is no cure for MS at the moment, but there are various therapy options to control symptoms, halt disease progression, and enhance quality of life (Wegner et al., 2013). Disease-modifying treatments (DMTs) and other drugs can help lessen the frequency and severity of relapses, while others target particular symptoms such as exhaustion, muscular stiffness, and pain (McGinley et al., 2021).

These are just a few examples of neurodegenerative disorders, and there are several others, each with its own specific characteristics and progression patterns. While treatments for these conditions are currently limited, ongoing research aims to better understand their causes and develop effective therapies to slow down or halt their progression

2.3 OXIDATIVE STRESS: MODULATOR OF NEURODEGENERATIVE DISORDER

Oxidative stress is thought to have an important role in the initiation and progression of neurodegenerative disorders. It denotes an imbalance among the generation of ROS and the body's ability to remove or repair the harm produced by them (Singh et al., 2019). ROS, including hydroxyl radicals, hydrogen peroxide, and superoxide radicals are natural by-products of regular cellular metabolism that are normally maintained in balance by the body's antioxidant defence system. However, under some situations, such as ageing, chronic inflammation, mitochondrial malfunction, or exposure to environmental pollutants, the body's antioxidant capacity might be exceeded, resulting in oxidative damage (Esmaeili et al., 2022). Oxidative stress may trigger inflammation in the brain. The activation of glial cells, such as microglia and astrocytes, causes the production and release of chemokines, reactive nitrogen

species, and pro-inflammatory cytokines (Liu et al., 2017). This prolonged inflammation exacerbates oxidative stress and contributes to brain damage and degeneration. ROS may directly damage DNA, causing strand breakage, base alterations, and the production of DNA adducts. This DNA damage can hamper DNA repair systems and contribute to genomic instability. Accumulated DNA damage can impair important cellular functions and contribute to neurodegenerative diseases (Teleanu et al., 2022).

ROS in cells can harm DNA, proteins, and lipids. Protein oxidation and DNA damage can both affect normal cellular function and lead to neuronal death. The mitochondrial complex is the primary source of ROS within cells. Oxidative stress can affect function of mitochondria, resulting in increased ROS generation and a continuing oxidative damage process. Mitochondrial dysfunction is particularly relevant in neurodegenerative disorders due to the high energy demands of neurons.

Oxidative damage can trigger an inflammatory cascade response in brain. Chronic inflammation attributes to neurodegeneration by promoting the release of inflammatory molecules and activating immune cells that can damage neurons. Protein misfolding and aggregation can be caused by oxidative stress, such as α-Syn in PD or tau and β-amyloid in AD. These protein aggregates are harmful to neurons and contribute to the course of illness. Antioxidant enzymes including glutathione peroxidase, catalase, and superoxide dismutase are frequently reduced in neurodegenerative diseases. This decrease in antioxidant capability worsens oxidative damage.

In the case of neurological disorders, oxidative damage plays a role in the progression of neurodegenerative illnesses such as PD, Prion disease, ALS, Huntington's disease, Multiple sclerosis, and AD. The brain is particularly vulnerable to oxidative injury due to its high lipid content and weak antioxidant defences. Oxidative stress in neurodegenerative disorders can have several detrimental effects:

(a) Protein Misfolding and Aggregation:

Protein misfolding and aggregation are closely related with oxidative stress in the context of neurodegeneration. Oxidative damage leads to the generation of ROS that can directly modify proteins (Soto and Pritzkow, 2018). ROS can oxidize amino acid residues within proteins, such as methionine, cysteine, and tyrosine, altering their chemical properties.

These oxidative modifications can disrupt protein folding and stability, increasing the likelihood of protein misfolding. Oxidative stress can disrupt the cellular machinery responsible for maintaining protein homeostasis, which includes protein folding, quality control, and degradation systems (Chopra et al., 2022). Molecular chaperones, which assist in proper protein folding, can be impaired by oxidative damage, compromising their ability to prevent misfolding. Additionally, oxidative stress can disrupt the function of the ubiquitin-proteasome system and autophagy, which are responsible for clearing misfolded proteins. Consequently, the accumulation of misfolded proteins occurs due to impaired protein clearance mechanisms. Misfolded proteins, particularly those resistant to degradation, can aggregate and form insoluble protein aggregates or fibrils as demonstrated in **Figure 2.5** (Gandhi et al., 2019).

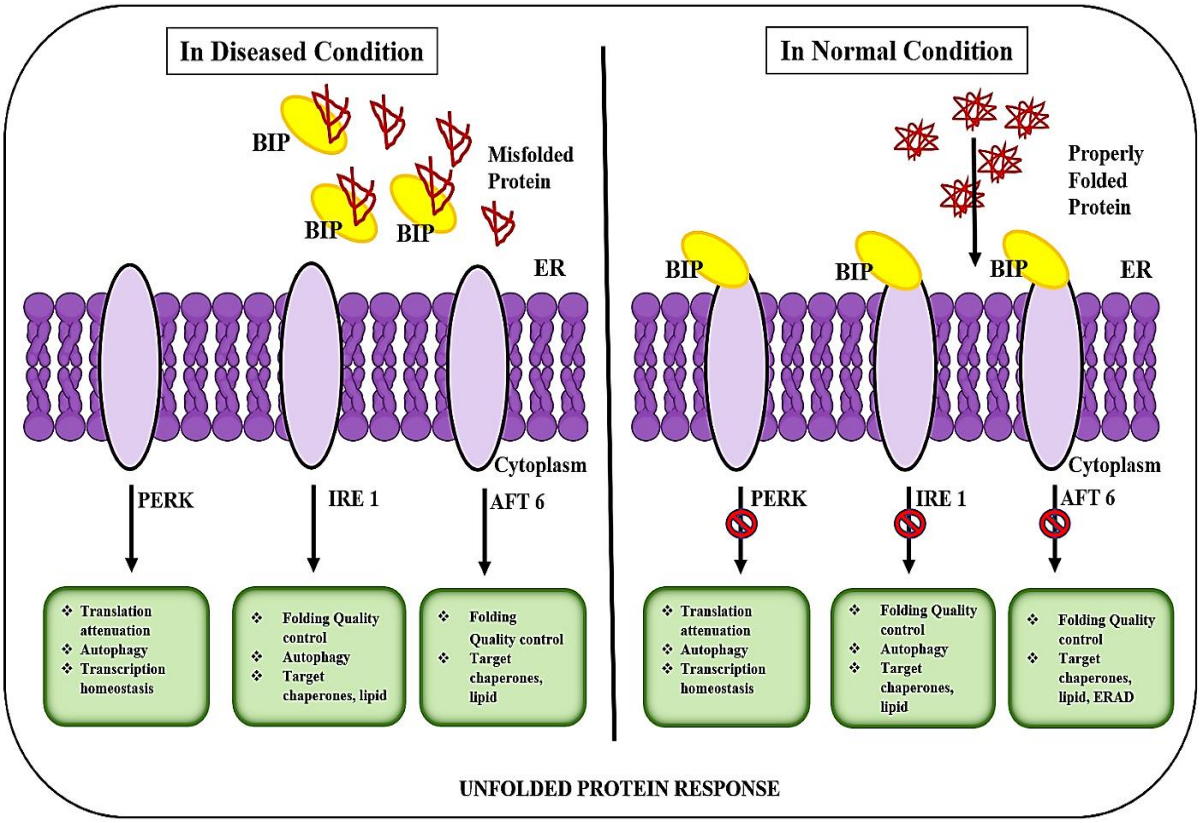


Figure 2.5: Unfolded Protein Response (UPR) In Diseased And Normal Conditions: The Unfolded Protein Response (UPR) is a cellular stress response system that is initiated in the endoplasmic reticulum (ER) in response to the buildup of misfolded or unfolded proteins. In the cell, the ER is in charge of protein synthesis, folding, and processing. Under normal circumstances BiP (chaperone) is normally linked with transmembrane proteins PERK1, ATF6, IRE1. Under disease circumstances, the sensors become active, resulting in the transcription of genes that control transcription, translation, and chaperones. BiP also attaches to misfolded proteins, causing them to refold (Chopra et al., 2022).

These aggregates can have a beta-sheet-rich conformation and are often referred to as amyloid aggregates. The process of protein aggregation is driven by intermolecular interactions between misfolded protein molecules, resulting in the production of larger aggregates. These protein aggregates are a hallmark feature of several neurodegenerative disorders, including PD, HD, and AD. Protein aggregation build-up can have a negative impact on cellular function and viability. These aggregates can disrupt normal cellular processes, impair cellular trafficking, interfere with proteostasis, induce cellular stress responses, and trigger inflammatory responses (Scannevin et al., 2018).

Furthermore, protein aggregates can directly interact with cellular components, including membranes and organelles, causing cellular dysfunction and leading to neuronal damage and death. It is vital to remember that the presence of protein aggregates can potentially cause oxidative stress. The aggregation process itself can generate ROS through various mechanisms, such as mitochondrial dysfunction and activation of inflammatory pathways (Tsoi et al., 2023). These ROS can further exacerbate oxidative stress, leading to a self-perpetuating cycle of protein misfolding, aggregation, and oxidative damage. The unfolded protein response (UPR) is a cellular homeostasis process. The UPR is triggered under stressful situations, such as AD. The ER membrane includes three sensors: PERK, IRE1, and ATF6. These proteins are coupled to another protein known as BiP, which inactivates the proteins. When BiP disintegrates under stress, these proteins are activated, causing changes in the translation and transcription processes and preventing cellular damage, while BiP functions as a refolds proteins and molecular chaperone (Ciechanover and Kwon, 2015).

BiP/GRP78 protein levels are raised in Alzheimer's disease, where they aid in protein refolding. The transmembrane protein PERK combines and phosphorylates the translation initiation factor eIF2, preventing translation. PERK is also involved in the activation of ATF4, which promotes the expression of chaperones. ATF4 expression over a prolonged period of time is connected to apoptosis (Ashraf et al., 2014). IRE1 homodimerizes and experiences a conformational shift, which activates the RNase domain in the protein's cytoplasm and controls the production of XBP-1, a transcription factor that transactivates genes associated to UPR proteins. Duran et al. (2017) revealed that IRE1 deletion lowered APP expression in a mouse model, indicating a role for IRE1 in the etiology of NDs. Overall, oxidative stress contributes to aggregation and protein misfolding in neurodegenerative diseases as mentioned in **Table 2.1** (Ochneva et al., 2022).

Table 2.1: Aggregation and misfolding of protein associated with neurodegenerative diseases.

S.No.	Aggregated protein(s)	Neurodegenerative Diseases	Affected areas of the brain	Clinical hallmarks	Cellular aggregate locations	Mode of Transmission	References
1.	α-synuclein	Parkinson Disease, Multiple System Atrophy, Dementia with Lewy Body	Hypothalamus, substantia nigra	Movement disorder	Cytoplasmic	Rarely inherited, mostly sporadic	(Goedert, 2001)
2.	Tau	Alzheimer Disease, Parkinson Disease, Fronto-temporal Dementia with Parkinsonism	Hippocampus, cerebral cortex	Progressive dementia	Extracellular, cytoplasmic	Inherited (6%) or sporadic (94%)	(Spillantin and Goedert, 2013)
3.	Amyloid-β	Alzheimer Disease	Cerebral cortex, hippocampus	Progressive dementia	Cytoplasmic, extracellular	Inherited (5%) or sporadic (95%)	(Bourdenx, et al., 2017)
4.	Superoxide dism utase (sod)	Amyloid Lateral Sclerosis	Brainstem, motor cortex	Movement disorder	Cytoplasmic	Inherited (9%) or sporadic (91%)	(Sanghai and Tranmer, 2021)
5.	Huntingtin	Huntington, Tar DNA-binding protein 43 (TDP-43)	Striatum, cerebral cortex	Dementia, psychiatric and motor problems	Nuclear	Autosomal dominated (Inherited)	(Koyuncu et al., 2017)
6.	Prion	Cretzfeld – Jakob Disease	Depending on the disease, different regions are involved.	Ataxia, insomnia, dementia, or psychiatric problems	Extracellular	Infectious (3%), Inherited (7%) or sporadic (89%)	(Baldwin and Correll, 2019)

(b) Lipid Peroxidation

A lipid peroxidation is an event that happens when extremely reactive chemicals, such as free radicals, destroy and harm lipids (fats) in the membranes of cells (Reed, 2011). It is one of the fundamental mechanisms behind oxidative damage, which is defined as an imbalance among free radical formation and antioxidant defences in the body. Various medical illnesses, including neurological diseases, have been linked to oxidative damage and lipid peroxidation. Oxidative stress has a crucial role in the evolution of neurodegenerative illnesses such as AD, PD, and ALS (Pea et al., 2019). The brain is especially prone to oxidative damage due to its rapid metabolic rate, substantial lipid content, and relatively limited antioxidant defences, as seen in **Figure 2.6**. Peroxidation of lipids can have numerous negative impacts on neurodegeneration.

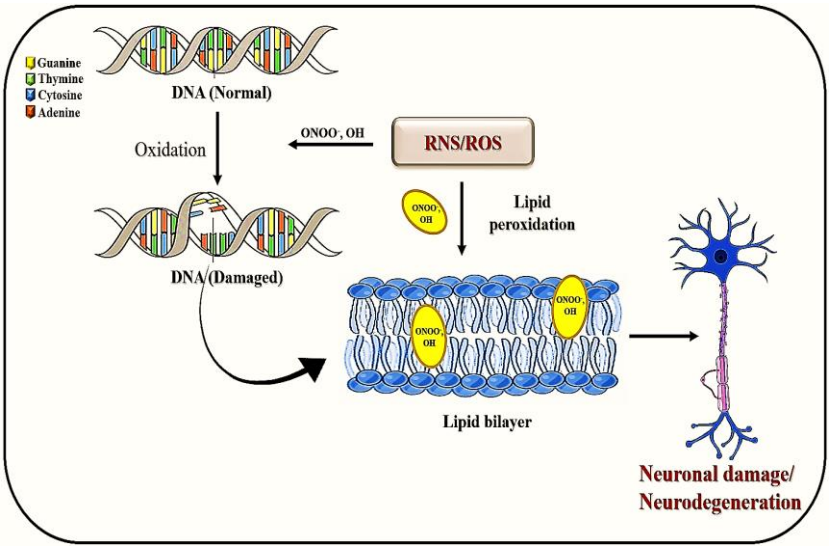


Figure 2.6: Reactive oxygen species including superoxide radicals ($O2^{\cdot -}$) and hydroxyl radicals ($\cdot OH$), can be generated during normal cellular metabolism or under conditions of oxidative stress. These ROS can directly interact with polyunsaturated fatty acids in membrane of cell, abstracting a hydrogen atom from the fatty acid chain and forming a lipid radical which leads to neurodegeneration.

Lipids are important components of cell membranes, and peroxidation disrupts their structure and function. This can compromise the integrity of neuronal membranes, leading to impaired cell signaling, altered ion transport, and increased permeability (Sultana et al., 2013). Lipid peroxidation generates reactive aldehyde by-products, such as MDA and 4-HNE.

These aldehydes are highly reactive and can modify proteins and nucleic acids, leading to DNA damage and cellular dysfunction (Angelova et al., 2021). Reactive aldehydes generated during lipid peroxidation can directly interact with DNA, forming adducts and DNA-protein cross-links. This DNA damage can interfere with DNA replication, transcription, and repair mechanisms, contributing to genomic instability and neuronal dysfunction. Lipid peroxidation can further enhance oxidative stress by generating additional ROS through reactions with transition metal ions or by promoting mitochondrial dysfunction (Villalón et al., 2022).

Mitochondria, the powerhouses of cells, are particularly susceptible to oxidative stress. The oxidation of lipids can compromise the membrane of mitochondria function, leading in energy loss, raised generation of reactive oxygen species, along with additional oxidative damage. Endogenous antioxidant systems in cells include enzymes such as superoxide dismutase and glutathione peroxidase, which aid in the elimination of ROS. In neurodegenerative diseases, the antioxidant defense systems may become compromised, leading to an accumulation of ROS and subsequent lipid peroxidation (Kinghorn et al., 2015).

Peroxidation of lipid metabolites can activate immune cells and promote the production of cytokines that are pro-inflammatory in the brain, causing inflammation. Furthermore, oxidative stress can affect calcium homeostasis, resulting in excitotoxicity, which is the overactivation of certain receptors that can result in neuronal cell death. The buildup of oxidative damage caused by lipid peroxidation, as well as subsequent events, can contribute to the gradual loss of neurons and the onset of neurological disorders (Dong and Yong, 2012). Lipid peroxidation products such as MDA and 4-HNE can cause oxidative damage to components of cells such as proteins, DNA, and lipids. This oxidative damage has the potential to alter cellular function and accelerate the evolution of neurodegenerative disorders. As a result, lipid peroxidation caused by oxidative stress is assumed to be important in both the onset and progression of neurodegenerative disorders (Grösgen et al., 2010).

Therefore, strategies aimed at reducing oxidative stress and lipid peroxidation, as well as enhancing antioxidant defenses, have been explored as potential therapeutic approaches for these conditions. It is worth noting that research in this field is still ongoing, and while lipid peroxidation and oxidative stress are considered significant factors in neurodegeneration, they are part of a complex disease process involving multiple mechanisms.

(c) Mitochondrial Dysfunction

Mitochondria are essential cellular organelles involved in energy production, calcium homeostasis, and various other cellular processes. They play an important role in neuron function and are especially sensitive to oxidative stress, which has been linked to neurological disorders (Lin and Beal, 2006). The aetiology of neurological disorders is thought to be aided by oxidative stress-induced dysfunction of the mitochondria. Mitochondria are a primary producer of ROS within the cell, especially during the oxidative phosphorylation process. Under normal circumstances, a small percentage of electrons leak from the electron transport chain, resulting in the generation of superoxide radicals ($O2^-$) within the mitochondrial matrix.

However, under oxidative stress, a disproportionate amount of reactive oxygen species is produced, overpowering the antioxidant system's cellular defence (Wang et al., 2019). ROS produced during oxidative stress can affect mitochondrial components such as proteins, mtDNA, and lipids. Oxidative destruction of mtDNA can disrupt mitochondrial gene expression, resulting in impaired mitochondrial function. Mitochondrial protein oxidation can affect their structure and function, reducing enzymatic activity and ATP synthesis. Damage to mitochondrial lipids caused by oxidation can compromise mitochondrial membrane integrity, impacting membrane potential and transport activities (Bhat et al., 2015).

Reactive oxygen species can particularly damage and target electron transport chain elements, resulting in electron leakage and further ROS formation. This results in a continuing cycle of increased oxidative stress and mitochondrial malfunction. ETC complex failure can result in decreased ATP generation, reduced membrane potential, and disturbed calcium homeostasis (Hroudová et al., 2014). The mPTP, a non-specific channel in the mitochondrial inner membrane, can be activated by oxidative stress. Excess calcium absorption, ROS, and an altered redox state can all cause mPTP to open, resulting in the dissipation of the mitochondrial membrane potential and the release of pro-apoptotic substances from the intermembranes region, such as cytochrome c. This can cause apoptotic cell death and lead to degeneration (Petrozzi et al., 2007). Mitophagy is a biological mechanism that removes damaged or malfunctioning mitochondria. However, oxidative stress can disrupt the delicate equilibrium of mitochondrial fission and fusion, resulting in fragmentation of mitochondria and impaired mitophagy. The buildup of malfunctioning mitochondria can aggravate oxidative stress and mitochondrial dysfunction. Mitochondrial malfunction inhibits ATP generation, resulting in neuronal energy failure. Energy-dependent functions like as synaptic transmission and ion gradient maintenance are jeopardised. Furthermore, defective mitochondrial metabolism impacts the utilisation of glucose and other energy substrates, causing cellular bioenergetics to be disrupted (Islam, 2017).

Overall, oxidative stress-induced mitochondrial dysfunction in neurodegeneration involves a cascade of events, including ROS overproduction, oxidative damage to mitochondrial components, impaired ETC function, mPTP opening, disrupted mitophagy, and energy failure. Understanding and targeting these mechanisms may provide potential therapeutic strategies for mitigating mitochondrial dysfunction and slowing down or preventing the development of neurological diseases.

(d) Inflammation

Two factors contribute to neurodegeneration: inflammation and oxidative damage (Glass et al., 2010). Oxidative damage, defined as a disparity among the creation of ROS and the cellular antioxidant system, can trigger inflammatory responses in the brain, exacerbating neurodegenerative processes as depicted in **Figure 2.7** (Chitnis and Weiner, 2017). The processes through which oxidative damage promotes inflammation in neurodegeneration include many cascades.

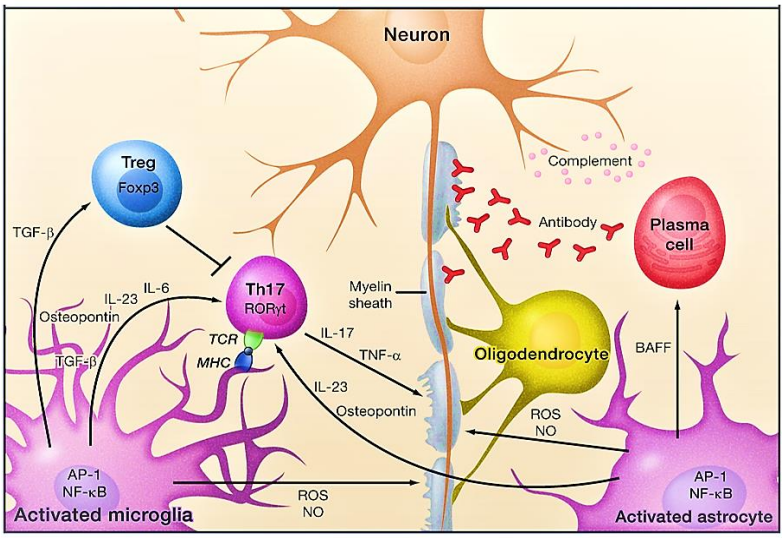


Figure 2.7: Oxidative stress can activate immune cells, particularly microglia and infiltrating immune cells in the CNS. Microglia, the resident immune cells in the CNS, can be activated by ROS and cytokines of pro-inflammatory. Activated microglia release pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), and produce ROS, amplifying the inflammatory response. Infiltrating immune cells, macrophages and including T cells, are also activated by oxidative stress and contribute to neuroinflammation (Glass et al., 2010).

Oxidative damage can activate transcription factors including activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB). These transcription factors regulate the expression of pro-inflammatory genes. NF-κB and AP-1 activation results in the generation of cytokines that are pro-inflammatory, chemokines, and other inflammatory mediators. Microglia, the resident immune cells in the CNS, and astrocytes, the supporting cells, play crucial roles in neuroinflammation (Walker et al., 2018). Microglia and astrocytes may be activated by oxidative stress, leading to their transition into pro-inflammatory phenotypes. Activated microglia produce cytokines that are pro-inflammatory, chemokines, and ROS, which exacerbate the inflammatory response. Astrocytes, when activated, contribute to the generation of mediators that promote inflammation, perpetuating neurological inflammation (Sun et al., 2023).

Oxidative damage can stimulate the generation of pro-inflammatory cytokines, such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6).

These cytokines promote inflammation by recruiting immune cells, increasing blood-brain barrier permeability, and activating glial cells. Oxidative stress can cause the oxidation of macromolecules such as proteins, lipids, and nucleic acids (Piancone et al., 2021). Through pattern recognition receptors (PRRs) expressed on immune cells, these changed biomolecules can behave as damage-associated molecular patterns (DAMPs) and induce inflammatory responses. Oxidised lipids and proteins, for example, can activate toll-like receptors (TLRs), triggering signalling cascades that increase inflammation (Simpson et al., 2020). The NLRP3 inflammasome, a multiprotein complex involved in the processing and production of pro-inflammatory cytokines IL-1 and IL-18, can be activated by oxidative stress-induced mitochondrial malfunction. Dysfunctional mitochondria produce mitochondrial DNA (mtDNA) and reactive oxygen species (ROS), which activate the NLRP3 inflammasome, leading in the production of cytokines that are pro-inflammatory and the worsening of neuroinflammation (Li et ed., 2020).

The interplay among inflammation and oxidative damage forms a vicious cycle, with oxidative stress promoting inflammation, and inflammation further exacerbating oxidative stress. This cycle contributes to the chronic neuroinflammation observed in various neurodegenerative disorders (Behl et al., 2021). Targeting oxidative stress and inflammation simultaneously represents a potential therapeutic approach for mitigating neurodegeneration.

(e) Neuronal Cell Death

Cell death of neurons in neurodegenerative illnesses is exacerbated by oxidative stress. The mechanisms through which oxidative damage attributes to death of neuronal cells in neurodegeneration includes cascades of process. Oxidative stress can cause damage to nuclear and mitochondrial DNA (mtDNA) through the oxidation of nucleic acids (Moujalled et al., 2021). Accumulation of DNA damage can impair DNA repair mechanisms and lead to genomic instability. Unrepaired or misrepaired DNA might cause the cell death cascade to be activated. Oxidative damage can result in the oxidation and misfolding of proteins within neurons. Oxidized and misfolded proteins can form aggregates and disrupt cellular function. Accumulation of aggregation of proteins, such as tau and amyloid-β in AD, can trigger neuronal cell death pathways.

Oxidative stress-induced lipid peroxidation can damage neuronal cell membranes (Park et al., 2020). Membrane disruption can result in disturbed ion homeostasis, reduced neural signalling, and amplification of cell death cascades. Peroxidation of lipid products, including 4-

hydroxynonenal (4-HNE), can also directly induce neuronal cell death through the production of ROS and the activation of apoptotic signaling pathways (Dang et al., 2022).

Oxidative damage can impair function of mitochondria and lead to mitochondrial dysfunction. Dysfunctional mitochondria generate higher level of ROS, leading in a further increase in oxidative damage. Mitochondrial dysfunction can compromise ATP production, disrupt calcium homeostasis, and stimulate the discharge of proteins that promote apoptosis including cytochrome c, triggering apoptotic cell death pathways (Filippone et al., 2022). Oxidative damage can initiate various cell death signaling pathways, including apoptosis, necrosis, and autophagy.

Excessive oxidative stress can disrupt the balance among pro-survival and pro-death signaling pathways, favoring the activation of cell death pathways. This can involve the activation of caspases, release of pro-apoptotic factors, and dysregulation of pro-survival signaling molecules (Ventruti and Cuervo, 2007). Oxidative stress-induced inflammation and excitotoxicity can attribute to cell death of neurons. Inflammatory mediators and excessive stimulation of glutamate receptors can cause excitotoxicity to neurons, resulting in the overactivation of cellular death processes. Persistent oxidative stress can activate planned death processes of cells such as necrosis or apoptosis, resulting in neuronal loss, a characteristic of neurological diseases (Moloudizargari et al., 2017).

These pathways work together to cause the gradual degeneration of neurons seen in neurological disorders. Understanding these systems might help identify new therapy options for reducing oxidative damage and preventing neuronal death in neurodegeneration.

(f) Cellular apoptosis

ROS mediated cellular apoptosis because of oxidative damage is a significant process in neurodegeneration. Oxidative stress occurs when the production of ROS exceeds the antioxidant defence systems in cells, therefore may culminate in cell death that is apoptotic (Cotman et al., 1995). Cascades of processes are involved in the pathways of ROS-mediated cellular death in dementia. Mitochondria are an important generator and target of ROS. Oxidative stress can cause mitochondrial dysfunction by damaging mitochondrial components such as proteins, DNA, and lipids (Ghavami et al., 2014). This malfunction interrupts the electron transport chain, affecting ATP synthesis and inducing electron leakage, which leads to increased ROS production. Abnormal ROS generation can induce additional mitochondrial destruction, resulting in the release of pro-apoptotic substances from the mitochondria into the

cytosol, such as cytochrome c. Cytochrome c initiates apoptotic cell death by activating caspase cascades (Tatton and Olanow, 1999). ROS can trigger a variety of signalling pathways that control apoptosis. ROS, for example, can activate cell-surface death receptors such as Fas receptor (CD95), tumour necrosis factor receptor 1 (TNFR1), and TNF-related apoptosis-inducing ligand receptors (TRAIL receptors). When ligands bind to these receptors, they create death-inducing signalling complexes (DISCs), which activate caspase-8 and cause apoptotic cell death (Zhou et al., 2019).

ROS can trigger oxidative effects on DNA, such as strand breakage, base alterations, and the production of DNA adducts. Severe DNA damage can activate the DNA damage response, which involves the activation of signaling kinases, such as ATM and ATR. The DNA repair mechanism can activate p53, a transcription factor that controls cell cycle arrest and death. Activated p53 can induce apoptosis by increasing the expression of pro-apoptotic genes such as Bax and Puma and decreasing the expression of anti-apoptotic genes such as Bcl-2 (Marks and Berg, 1999).

Caspases are key players in the execution of apoptosis. ROS can activate caspases through multiple mechanisms. ROS can directly oxidize and activate caspases, or they can modulate the activities of caspase regulators, such as Bcl-2 family proteins. ROS can disrupt the balance between pro-apoptotic and anti-apoptotic Bcl-2 family members, leading to the release of cytochrome c from the mitochondria, caspase-9 activation, and subsequent activation of downstream effector caspases, such as caspase-3. ROS can modulate anti-apoptotic pathways that normally protects cell from apoptosis (Stefani et al., 2012).

For instance, ROS can oxidize and inactivate thioredoxin, a key antioxidant protein that inhibits apoptosis by reducing disulphide bonds in pro-apoptotic proteins. Inactivation of thioredoxin disrupts its anti-apoptotic function, promoting apoptotic cell death. Overall, ROS-mediated cellular apoptosis due to oxidative stress in neurodegeneration involves mitochondrial dysfunction, activation of apoptotic signaling pathways, DNA damage, caspase activation, and disruption of anti-apoptotic pathways.

Understanding these mechanisms is crucial for developing strategies to mitigate oxidative stress and prevent or slow down neuronal apoptosis in neurodegenerative disorders as shown in **Figure 2.8** (Londono et al., 2012).

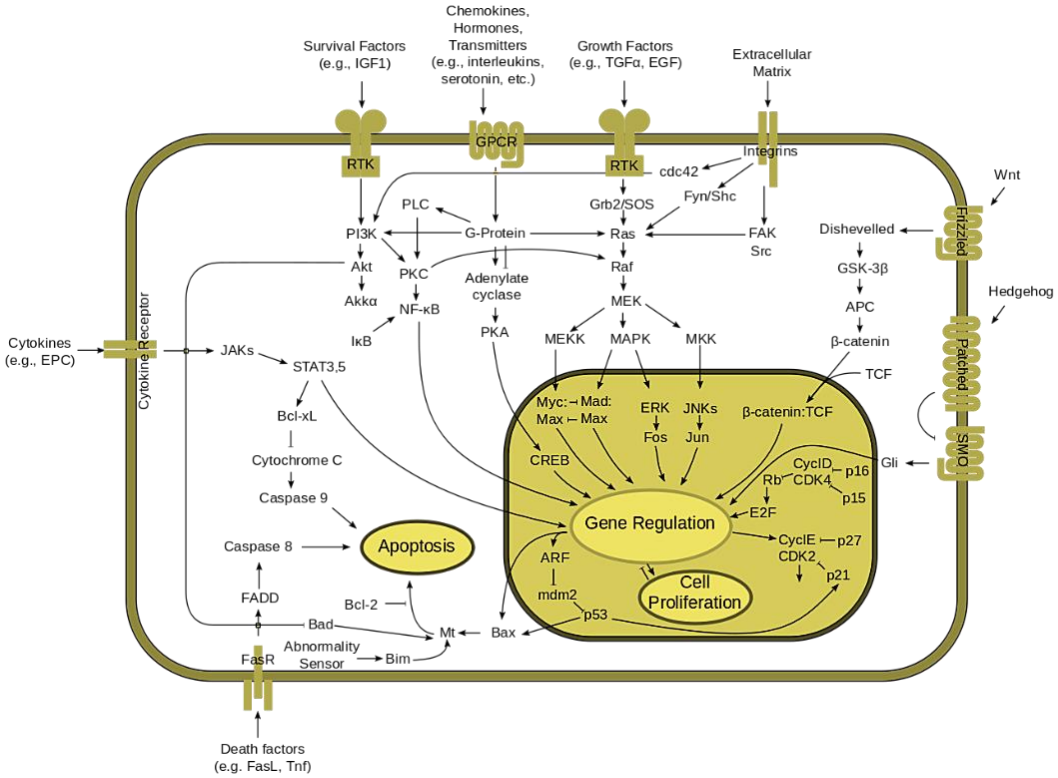


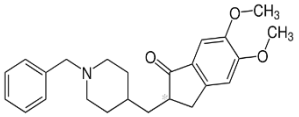
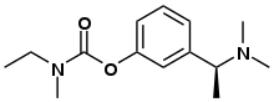
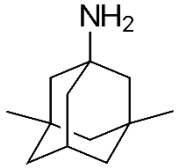
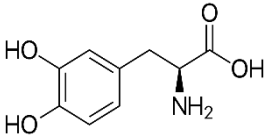
Figure 2.8: Oxidative stress can activate various stress-responsive signaling pathways, including p38 mitogen-activated protein kinase (MAPK) pathway and the c-Jun N-terminal kinase (JNK) pathway. These mechanisms induce necrosis by regulating pro-apoptotic gene expression and suppressing anti-apoptotic factors (Ghavami et al., 2014).

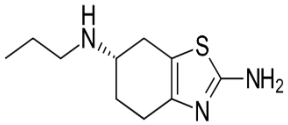
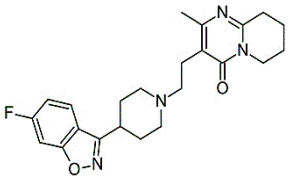
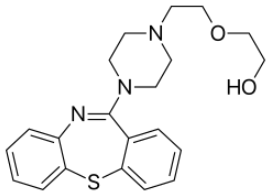
Given the importance of oxidative damage in neurological disorders, numerous treatment techniques targeted at lowering oxidative stress or increasing antioxidant defence systems have been investigated. More study is required to completely comprehend the complicated mechanisms at work and to design successful treatment strategies.

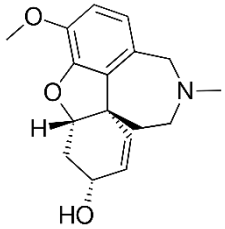
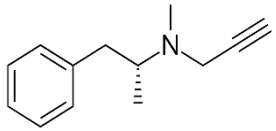
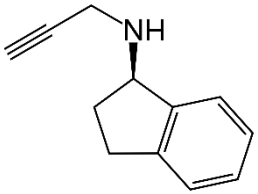
2.4 CONVENTIONAL MEDICINES USED FOR NEURODEGENERATION AND THEIR NEUROTOXIC EFFECTS.

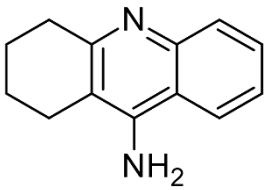
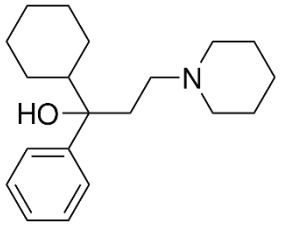
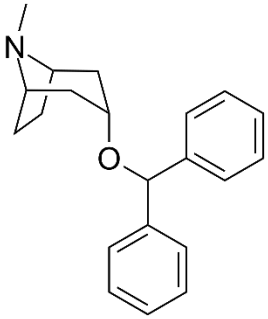
Systemic administration of medicines to the CNS is a substantial obstacle, owing to their short half-life, considerable first-pass metabolism, low access to the brain, and potential adverse effects when reaching non-target peripheral organs (Misra et al., 2003). Cholinesterase (ChE) inhibitors, tacrine, N-methyl-D-aspartate (NMDA) agonists in connection with Vitamin D, levodopa, or other dopaminergic agonists and memantine have never been utilised in conventional medicine or therapeutics to alleviate anything apart from motor symptoms by replenishing neurotransmitters (Mansor et al., 2019). Nevertheless, long-term use of these drugs can have serious adverse effects, including additional motor difficulties, as shown in **Table 2.2**.

Table 2.2: List of some conventional medicines used for neurodegeneration and their neurotoxic effects.

S.No	Drug	Molecular formulae	Neurotoxic effect	Clinical Uses	Chemical structure	Mol. weight (g/mol)	References
1.	**Donepezil**	$C_{24}H_{29}NO_3$	The most prevalent adverse events leading to clinical trial termination were bradycardia, confusion, and diaphoresis. Occasionally cause gastr-ointestinal side effects.	Donepezil binds to and inactivates cholinesterase, preventing acetylcholine breakdown. This raises the concentrations of acetylcholine at cholinergic synapses.		379.492	Saxena et al., 2008
2.	**Rivastigmine**	$C_{14}H_{22}N_2O_2$	A rivastigmine excess can cause seizures or shock. Large pupils, erratic breathing, and a quick, weak pulse are all symptoms of shock.	This drug works by inhibiting the breakdown of acetylcholine, a neurotransmitter involved in memory and cognitive function in some individuals with AD.		250.337	Gupta et al., 2021
3.	**Memantine**	$C_{12}H_{21}N$	Common side effects include dizziness, headache, confusion, and constipation. In rare instances, it may cause hallucinations, aggression, and increased confusion.	Memantine is an NMDA receptor antagonist used to manage moderate to severe Alzheimer's disease. It works by regulating the activity of glutamate, an excitatory neurotransmitter.		179.3	Rojas et al., 2008
4.	**Levodopa**	$C_9H_{11}NO_4$	The majority of the evidence for levodopa toxicity comes from *in vitro* experiments, which show that levodopa can harm dopaminergic neurons through a process that most likely includes oxidative stress.	Dopamine is a precursor of levodopa. Levodopa is most typically used as a dopamine replacement medication in the treatment of PD. It is particularly efficient in controlling bradykinetic symptoms seen in PD.		197.1879	Romagnolo et al., 2018

5.	**Pramipexole**	$C_{10}H_{17}N_3S$	Light-headedness, dizziness, or fainting, particularly when rising abruptly from a sitting or lying posture. observing, hearing, or experiencing things that are not present.	Used to control and treat Parkinsonism and restless leg syndrome. This exercise discusses the indications, action, and contraindications of pramipexole as a beneficial medication in the treatment of parkinsonism and restless leg syndrome.		211.324	Uberti et al., 2007
6.	**Risperidone**	$C_{23}H_{27}FN_4O_2$	Risperdal has also been linked to movement abnormalities that worsen with time. Even if the person quits using Risperdal, the abnormali-ties may persist.	Risperidone is prescribed to adults and teens 13 years of age and older to treat the symptoms of schizophrenia (a mental disorder characterised by abnormal or strange thinking and lack of interest in life).		410.485	Reeves et al., 2002
7.	**Quetiapine**	$C_{21}H_{25}N_3O_2S$	Cataracts, weight gain, high blood sugar, high cholesterol, and tardive dyskinesia, an uncommon disorder characterised by involuntary and abnormal movements of the jaw, lips, and tongue, are some of the symptoms.	This drug can help you focus and reduce hallucinations. It enables you to think more clearly and positively about yourself, to feel less frightened, and to participate more actively in daily life. It may also boost your mood, sleep, appetite, and vitality.		383.5099	He et al., 2005

8.	**Galantamine**	$C_{17}H_{21}NO_3$	Galantamine can induce nausea, vomiting, diarrhoea, lack of appetite, stomach discomfort, heartburn, weight loss, and excessive weariness. It will not cure Alzheimer's disease, nor will it slow its progression.	Galantamine is a cholinesterase inhibitor having a dual mode of action. It is a reversible inhibitor of acetylcholine esterase that increases the intrinsic action of acetylcholine on nicotinic receptors.		287.354	Kola et al., 2023
9.	**Selegiline**	$C_{13}H_{17}N$	The drug's anticholinergic properties are primarily responsible for the drug's common side effects of xerostomia and constipati-on. Other typical side effects include headaches, dizziness, sleeplessness, and nausea.	Selegiline is an MAO inhibitor, which is a kind of drug. This medicine is thought to assist inhibit the breakdown of dopamine in the brain. This medicine is typically used in conjunction with Sinemet at a later stage of Parkinson's disease.		187.2808	Ebadi et al., 2006
10.	**Rasagiline**	$C_{12}H_{13}N$	Rasagiline may produce minor headaches, joint or neck discomfort, heartburn, nausea, vomiting, stomach pain, constipation, and diarrhoea as side effects.	Rasagiline is used to treat the symptoms of Parkinson's disease (a slowly progressing nervous system disease characterised by a fixed face without expression, slowing of movements, and muscle weakness).		171.238	Bar et al., 2004

11.	**Tacrine**	$C_{13}H_{14}N_2$	With acute exposure, tacrine induces an increase in blood enzymes, indicating liver cell injury. According to research, the neurotoxic effects of tacrine in the brain are caused by a rise in ACh levels, which leads to overstimulation of muscarinic receptors.	It works by slowing the breakdown of acetylcholine. Used to treat Alzheimer's and dementia symptoms. This activity discusses the indications, mechanism of action, and contraindications for cholinest-erase inhibitors in the treatment of dementia problems.		198.264	Saxena et al., 2008
12.	**Trihexypheni dyl**	$C_{20}H_{31}NO$	Severe sleepiness, dilated pupils, fever, paleness in your face, and skin, hallucinations, paranoia, seizure, or numbn-ess in or around your mouth, throat, or nose are all indications of an overdose.	This drug helps to manage the tremors and rigidity associated with Parkinson's disease. They work by blocking the effects of acetylcholine, thereby restoring balance between acetylcholine and dopamine in the brain.		301.466	Saitoh et al., 1988
13.	**Benztropine**	$C_{21}H_{25}NO$	Benztropine possesses antichol-inergic as well as antihistaminic properties. The anticholinergic effects of benztropine are due to its blockage of the neurotransmitter acetylcholine. Symptoms may include dizzin-ess and difficulty thinking or seeing clearly.	Benztropine maintains the balance of acetylcholine and dopamine in the regions of the brain that govern muscular movement. This reduces the movement side effects induced by antipsychotic drugs and Parkinson's disease.		307.429	Sogawa et al., 2020

2.5 ROTENONE (NEUROTOXIC SUBSTANCE)

Rotenone is an organic compound that occurs naturally and is generated from the rhizomes of some plants, including the jicama vine and the barbasco plant (Bisbal and Sanchez, 2019). It has been used for various purposes throughout history, including as a natural insecticide and piscicide (a substance used to kill fish). It has also been employed in research to study the mechanisms of dementia, particularly in relation to PD (Fikry et al., 2022).

2.5.1 Mechanism of rotenone-induced neurotoxicity

The primary method of action of rotenone involves its suppression of the mitochondrial complex I, which is an essential element of the chain of electron transport responsible for energy production within the cell **(Figure 2.9)** (Pamies et al., 2018). Rotenone specifically targets and inhibits the activity of complex I in the mitochondria. Complex I is in charge of the electron transfer from NADH to coenzyme Q10 (CoQ10) during the process of oxidative phosphorylation, which creates the electrochemical gradient required for ATP generation. Rotenone disrupts the usual flow of electrons via the electron transport chain by blocking complex I. This disruption leads to decreased ATP production, as the energy derived from the transfer of electrons is reduced. Rotenone inhibits complex I, causing electron buildup in the electron transport chain (Wang et al., 2020). These stored electrons may interact with molecular oxygen, producing abundant ROS such superoxide anion and hydrogen peroxide . ROS are very reactive chemicals that can cause damage to biological elements such as lipids, proteins, and DNA.

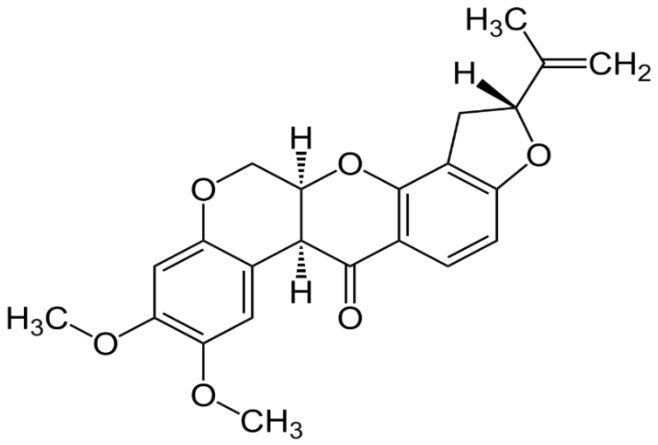


Figure 2.9: Chemical structure of rotenone.

Rotenone's enhanced generation of ROS causes oxidative stress within the cells. When there is an imbalance between the generation of ROS and the ability of cells to detoxify or repair the consequent damage, oxidative stress arises. Elevated ROS levels may incapacitate the cellular antioxidant defence mechanisms, resulting in oxidative damage to diverse cellular structures, as shown in **Figure 2.10.** (Bhurtel et al., 2019).

Rotenone's suppression of mitochondrial complex I and consequent oxidative stress can further impair mitochondrial function. The impaired mitochondrial function caused by rotenone contributes to the overall neurodegenerative process (Adedara et al., 2023). The exact reasons for this vulnerability are unknown, although variables that include the reliance on mitochondrial function for dopamine synthesis and the existence of higher levels of oxidative damage in these neurons may contribute to their increased sensitivity (Rao et al., 2019).

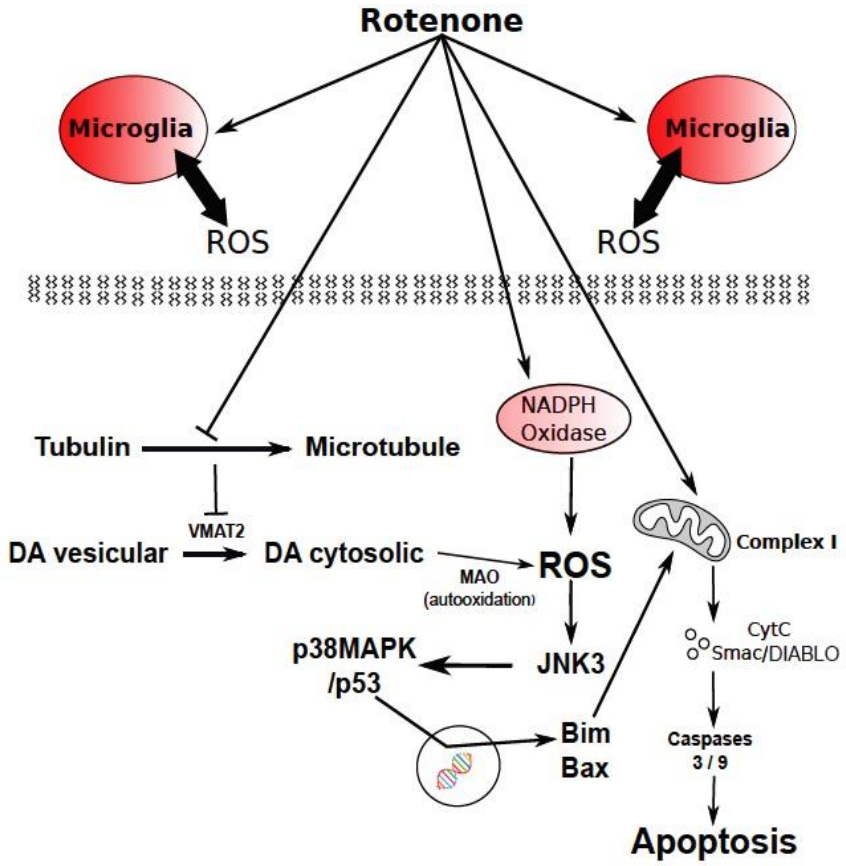


Figure 2.10: Schematic representation of apoptotic pathways in neurodegeneration due to rotenone. Rotenone promotes the production of reactive oxygen species (ROS) in the cytoplasm by a variety of methods, that include: (1) depolymerization of microtubules. When microtubules depolymerize, vesicular transport of dopamine (DA) is interrupted, leading in an increase in cytosolic DA concentration and an increase in ROS levels owing to DA autooxidation produced by monoamine oxidases (MAO). (2) By suppressing complex I of mitochondrial chain, and (3) by activating various enzymes that create ROS, including the nicotinamide adenine dinucleotide phosphate (NADPH)-oxidases (Heinz et al., 2019).

The link between rotenone and neurodegeneration has been extensively studied, particularly in relation to Parkinson's disease. It can induce Parkinson's disease-like symptoms and pathological changes in animal models, affecting mitochondrial function, leading oxidative damage, and promoting the expression of alpha-synuclein. More study is required to completely comprehend the mechanisms and to design successful treatment strategies.

2.6 IMPORTANCE OF MEDICINAL PLANTS AND THEIR THERAPEUTIC APPLICATIONS

Medicinal plants have traditionally been utilised by humans as a traditional way of treating a variety of diseases. Despite significant improvements in therapeutic development, there is still a need for effective and potent analgesic drugs (Radha et al., 2021). In this context, it has been widely described that numerous plant-derived compounds serve an essential part in the process of developing novel techniques to treat various diseases. Recent research on herbal plants or medicine has resulted in significant advances in the pharmacological assessment of diverse plants utilized in traditional medical systems (Pathak et al., 2022). Secondary metabolites or substances found in medicinal plants include tannins, terpenoids, alkaloids, and flavonoids, which determine the therapeutic effectiveness of the plant, particularly its antioxidant activity. Secondary metabolites are very diverse low molecular weight substances with a wide range of biological characteristics that interact with proteins, nucleic acids, and other biomembranes and are active and volatile targets of cells (Siddiqui et al., 2022).

Herbal remedies play a crucial part in healthcare and have various therapeutic applications. Here are some key reasons highlighting the importance of medicinal plants and their therapeutic applications:

- ***Traditional Medicine:*** For ages, medicinal plants have been employed in traditional medical systems including Ayurveda, Traditional Chinese Medicine, and Indigenous healing practises. They form the foundation of these systems and have been trusted for their healing properties by communities worldwide (Keihanian et al., 2022).
- ***Rich Source of Bioactive Compounds:*** Medicinal plants contain numerous bioactive compounds including flavonoids, alkaloids, terpenoids, and tannins, which possess medicinal properties. These compounds can have diverse impacts on the human health, including analgesic, anti-inflammatory, antimicrobial, antioxidant, anticancer, and antidiabetic activities (Zhu et al., 2022).
- ***Drug Discovery and Development:*** Many modern pharmaceutical drugs are derived from or inspired by medicinal plants. Natural plant substances serve as the foundation for the creation of novel drugs. Examples include the use of the plant-derived compound paclitaxel for cancer treatment and the development of aspirin from willow bark (Dutra et al., 2016). Medicinal herbs are often the primary source of healthcare in many developing countries, where access to conventional medicine may be limited.

Local communities rely on traditional herbal remedies derived from medicinal plants for treating common ailments and maintaining their well-being (Barkat et al., 2021). Medicinal plants are an integral part of complementary and alternative medicine practices. Many people seek natural and plant-based treatments as alternatives or supplements to conventional medicine. Herbal remedies and supplements made from medicinal plants are used for various purposes, including immune support, stress reduction, and overall wellness (Jahangir et al., 2020).

- ***Nutraceuticals and Functional Foods:*** Medicinal herbs are also used to create functional meals and nutraceuticals. These products go beyond basic nourishment to give additional health advantages. They are created with specialised plant extracts or bioactive chemicals to promote various areas of health, such as cardiovascular health, cognitive function, and digestive wellness (Ong, 2004).
- ***Conservation and Biodiversity:*** The preservation and sustainable utilization of medicinal plants contribute to biodiversity conservation efforts. By promoting the conservation of natural habitats and ensuring the sustainable harvesting of medicinal plants, we can protect valuable plant species and maintain the delicate ecological balance (Dey et al., 2019).
- ***Research and Innovation:*** Ongoing scientific research on medicinal plants helps validate their traditional uses, discover new applications, and optimize their effectiveness (Tiwari et al., 2016).

Curcuma longa, Zingiber officinale, and *Aloe barbadensis* have anti-inflammatory properties that can help to reduce inflammation in the body (Venkatadri et al., 2020). *Echinacea purpurea* and *Allium sativum* contain natural compounds that can help fight bacteria, viruses, and fungi. Some medicinal plants have analgesic properties that can help alleviate pain including *Salix alba*, which contains salicylic acid (the active ingredient in aspirin), and *Zingiber officinale*, which contains compounds that can reduce pain and inflammation (Sharma et al., 2022). Many medicinal plants including *Mentha piperita and Matricaria chamomilla* can promote digestive health by reducing inflammation and supporting the growth of healthy gut bacteria. Some medicinal plants have calming and relaxing properties that can help reduce anxiety and stress. Examples include *Matricaria chamomilla* and *Passiflora incarnata* (Wang et al., 2020).

Numerous studies in the world have confirmed the beneficial effects of *Urtica dioica*, *Matricaria chamomilla*, and *Murraya koenigii*. Understanding the molecular mechanisms contributing to beneficial outcomes can pave the way for novel treatment approaches.

2.6.1 Urtica dioica

2.6.1.1 Botanical Properties: *Urtica dioica*, also referred to stinging nettle, is an *Urticaceae* family perennial herbaceous plant (**Figure 2.11)**. It is native to Asia, Europe, Northern Africa, and North America and can be found in various regions around the world (Grauso et al., 2020). For generations, UD extract has been utilised for its therapeutic and nutritional benefits. *U. dioica* is a herbaceous plant that can reach a height of 1-2 metres (3-6 feet). It has a spreading habit and forms dense clumps or patches. The stem is erect, four-sided, and covered with stinging hairs. The leaves of stinging nettle are opposite, toothed, and ovate in shape. They are characterized by fine hairs, including stinging hairs, which contain irritants that cause a stinging sensation when touched (Dhouibi et al., 2020). The leaves have a dark green color and prominent veins. The flowers lack petals and are instead surrounded by four greenish sepals. Stinging nettle is notorious for its stinging hairs, which act as a defense mechanism. These hairs contain chemicals, such as formic acid and histamine, that cause a stinging and itching sensation when they come into contact with the skin. Stinging nettle contains a variety of bioactive compounds that contribute to its medicinal properties (Devkota et al., 2022). These include flavonoids, phenolic acids, lectins, vitamins (that include vitamin C and vitamin K), minerals (that include iron and silica), and various other nutrients. It has been utilized to treat conditions like sensitivities, joint inflammation, skin aggravations, and urinary tract issues. Stinging nettle is also used as a nutritive tonic and is believed to support overall health and well-being (Zhang et al., 2014).

2.6.1.2 Taxonomic Classification of *Urtica dioica:*

Kingdom: Plantae

Phylum: Magnoliophyta

Class: Magnoliopsida

Order: Urticales

Family: Urticaceae

Genus: *Urtica*

Species: *Urtica dioica L.*

Scientific name: *Urtica dioica*

Common name: Stinging nettle

Figure 2.11: *Urtica dioica*

2.6.1.3 Phytochemical Properties: *Urtica dioica* contains several phytochemicals that contribute to its medicinal properties. It contains various flavonoids, including quercetin, kaempferol, and rutin which are recognised for their anti-inflammatory and antioxidant benefits. They also have potential anti-allergic, anti-cancer, and neuroprotective properties. Lectins are carbohydrate-binding proteins found in *U. dioica*. They have been proven to have immunomodulatory characteristics and might possess anti-inflammatory capabilities. *U. dioica* contains sterols, such as beta-sitosterol (Dar et al., 2013). These substances have been associated with immunomodulatory and anti-inflammatory properties. There are other phenolic chemicals in this plant, such as ferulic acid, chlorogenic acid, and caffeic acid. These chemicals have antioxidant properties and contribute to the plant's anti-inflammatory properties. Triterpenes such as oleanolic acid and ursolic acid are found in Urtica dioica. Antiinflammatory, antioxidant, anti-cancer, and hepatoprotective activities have been demonstrated for these substances (Gulcin et al., 2004). *Urtica dioica* seeds contain essential fatty acids, including omega-3 and 6, and minerals including calcium, magnesium, potassium, and iron. It also contains vitamins, including vitamin C and β-carotene, which have antioxidant properties and support overall health .

These phytochemicals present in *Urtica dioica* contribute to its potential medicinal properties and have been studied for their effects on inflammation, allergies, prostate health, and other conditions (Bourgeois et al., 2016).

2.6.1.4 *In-vivo* Pharmacological studies on *Urtica dioica:*

(a) Anti-inflammatory effects: *Urtica dioica* contains phyto-constituents, including phenolic constituents and flavonoids, which have been demonstrated to be anti-inflammatory. Chronic inflammation is often associated with neurodegenerative diseases, and reducing inflammation may help protect neurons from damage. Scientific study has emphasised the nettle's capacity to reduce the inflammatory response via several routes, with the end result being a reduction in the creation of lipid mediators and proinflammatory cytokines. Leaf fractions reduce the development of thromboxanes and prostaglandins by inhibiting the formation of arachidonic acid pathway enzymes, including the cyclo oxygenases COX-1 and 2 (Chira et al., 2022).

(b) Antioxidant activity: *Urtica dioica* exhibits antioxidant activity, which can help counteract oxidative stress. Antioxidants assist to neutralize free radicals and prevent cellular damage, which is important in the evolution of neurological conditions. Nettle extracts are capable of neutralising reactive oxygen species (ROS). Their antiradical activity against superoxide anion

O2, OH, and NO was evaluated by spectroscopy. Numerous studies have shown that leaf fractions in ethanol and methanol have a high antioxidant effect on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) (Jaiswal and Lee, 2022).

(c) Antidiabetic activity: A research on diabetic rats found that aqueous extracts of nettle leaves had a hypoglycaemic effect. The reduction of intestinal glucose absorption supports these results. Furthermore, research on the islets of Langerhans have shown that nettle has a stimulatory effect on insulin release, which is followed by a drop in blood sugar. This conclusion was confirmed by tests performed on normal and diabetic rats following intraperitoneal administration of aqueous extracts (Tabrizi et al., 2022).

(d) Antiulcer properties: Nettle prevents stomach ulcers in a dose-dependent manner. Aqueous fraction of aerial parts prevented rats against stomach ulcers at doses of 50 and 200 mg/kg, with protective rates ranging from 68.6 to 76.7% (Sisay et al., 2021).

(e) Cholinergic activity: *Urtica dioica* has been shown to have cholinergic activity, which means it may have an effect on the system of neurons in the brain. The cholinergic system is essential for cognitive function, and disruption of this system is shown in neurodegenerative illnesses including AD (Patel et al., 2015).

(f) Antihypertensive properties: Intravenous injections of an aqueous fraction of U. dioica aerial parts at two dosages (4 and 24 mg/kg/h) resulted in blood pressure decreases of 16% and 39%, respectively, proportional to the given dose. This decrease was associated with an increase in natriuresis. However, when a low dosage (4 mg/kg/h) was administered, the hypotensive effect was reversible after one hour, but it maintained when a substantial amount (24 mg/kg/h) was utilised (Qayyum et al., 2016).

(e) Diuretic activity: *Urtica dioica* has diuretic properties, meaning it can increase urine production and promote the excretion of excess water and waste products from the body. Previous study reported that diuretic effect may be beneficial in conditions such as edema and urinary tract infections (Taheri et al., 2022).

While *Urtica dioica* has shown promise in preclinical trials, further study is required to fully comprehend its impact on neurodegeneration and to determine its safety and effectiveness in human patients.

2.6.2 Matricaria chamomilla

2.6.2.1 Botanical Properties: Chamomile, often known as *Matricaria chamomilla*, is a small Asteraceae flowering plant **(Figure 2.12)**. It is native to Western Asia and Europe, but has spread to other regions of the world (Singh et al., 2011). Chamomile is an annual herbaceous plant that matures to a height of around 15-60 cm (6-24 inches). It has a branching, erect stem covered with fine, feathery leaves. The leaves of chamomile are alternate, bipinnate, and have a fern-like appearance. They are divided into thread-like segments, giving them a feathery appearance. The flowers of chamomile are small and daisy-like, with a yellow center surrounded by white petals. They are borne on long stalks and have a pleasant, apple-like fragrance. The flowers bloom in late spring to early summer (Farzadfar et al., 2013).

Chamomile contains several bioactive compounds that contribute to its medicinal properties. The main constituents include essential oils (such as chamazulene, bisabolol, and farnesene), flavonoids (such as quercetin, apigenin, and luteolin), and other beneficial compounds. It has anti-inflammatory, antioxidant, and antimicrobial effects as well.

Chamomile flowers can be used to make herbal tea, which is popular for its soothing and calming effects. The tea is often consumed to promote relaxation and relieve stress. While chamomile is primarily known for its calming and sedative effects, it also possesses several potential benefits that may be relevant to neurodegeneration (Avallone et al., 2000).

2.6.2.2 Taxonomic Classification of *Matricaria chamomilla*

Kingdom: Plantae
Phylum: Magnoliophyta
Class: Magnoliopsida
Order: Asterales
Family: Asteraceae
Genus: *Matricaria*
Species: *chamomilla*
Scientific name: *Matricaria chamomilla*
Common name: Chamomile

Figure 2.12: *Matricaria chamomilla*

2.6.2.3 Phytochemical Properties: Chamomile contains a diverse range of phytochemicals, which are naturally occurring compounds that contribute to its therapeutic properties. Chamomile is rich in flavonoids, including apigenin, luteolin, quercetin, and their glycosides (Mihyaoui et al., 2022). Flavonoids possess antioxidant, antimicrobial, and anti-inflammatory, abilities, this can help protect the body from oxidative stress, decrease inflammation, and resist infections. *Matricaria chamomilla* contains terpenoids such as chamazulene, α-bisabolol, bisabolol oxide, and matricin which exhibits anti-inflammatory, soothing and skin-calming properties. Chamomile also contains coumarins like herniarin and umbelliferone which have anticoagulant and vasodilatory properties, and may contribute to chamomile's ability to promote blood circulation and relieve muscle spasms (Baghalian et al., 2011). Chamomile contains phenolic acids including p-coumaric acid, chlorogenic acid, and caffeic acid which are responsible for anti-cancer and antioxidant effects. *Matricaria chamomilla* contains various glycosides, apigenin and luteolin are two examples. Chamomile's anti-inflammatory and antioxidant properties are enhanced by these glycosides. (Mailänder et al., 2022).

The combination of these phytochemicals in *Matricaria chamomilla* is believed to contribute to its several medicinal qualities, including anti-inflammatory, antimicrobial, antioxidant, sedative, and digestive benefits.

2.6.2.4 *In-vivo* Pharmacological studies on *Matricaria chamomilla*

Anti-inflammatory properties: Chamomile contains compounds, such as chamazulene, apigenin, and bisabolol, which have anti-inflammatory properties. The volatile and non-volatile components of chamomile, essential oil and aqueous fraction, may all strongly decrease xylene-induced edoema of mouse ears, and carrageenan-induced pedal swelling in rats, according to prior research.

They also demonstrated a considerable inhibitory effect on carrageenan-induced increases in prostaglandin E2 and nitric oxide levels in rat pedal edoema (Biltekin et al., 2023). The effects of an M. chamomilla hydroalcoholic extract on inflammatory blood marker levels in rats. Blood levels of TNF, IL-6, and fibrinogen were considerably lower after treatment with 109 mg/kg hydroalcoholic extract (Shebbo et al., 2020)

(a) Antioxidant effects: Chamomile contains flavonoids and other compounds that possess antioxidant properties, helping to decrease oxidative damage and eliminate free radicals to cells. According to Singh et al. (2011), chamomile fraction demonstrated strong antioxidant qualities in lipid peroxidation studies using chicken liver tissue. MC fraction decreased the

rise in SOD globules and plasma malondialdehyde caused by an elevated cholesterol feed in male Wistar rats. Furthermore, 10 and 20% chamomile powder significantly decreased lipid peroxidation while raising catalase, glutathione levels, and acetylcholine esterase in diabetic rats (Kolodziejczyk et al., 2015).

(b) Antidiabetic properties: Anti-amylase and maltase activities of MC fraction and extracted glucosides apigenin, cis and trans-2-hydroxy-4-methoxycinnamic acid were investigated. The results indicate that both the portion and the drugs hindered the activity of enzymes in a dependent on concentration way. Apigenin and apigenin-7-O-glucoside inhibited maltase and α-amylase the most.

Another study discovered that MC hydro-methanolic fraction and many isolated components decreased the activity of rat lens aldose reductase. Furthermore, in high-glucose conditions, 3,5-O-di-caffeoylquinic acid and luteolin-7-O-β-d-glucuronide reduced sorbitol aggregation in rat lens, but luteolin and luteolin-7-O-β-d-glucuronide hindered the formation of advanced products of glycation. Furthermore, the MC ethanolic extract demonstrated anti-glycation properties as well as lipase inhibiting properties with an IC_{50} of 259.4 g/ml (Cemek et al., 2008).

(c) Anti-tumoral properties: *Matricaria chamomilla* extracts and essential oils have also been examined for their anti-tumoral activities on a wide range of cancer cell lines. Chamomile extract was tested for anticancer activity on a human breast carcinoma (MCF-7) cell line. It reduced cell growth in a dose-dependent manner, with 88.9% suppression after 24 hours at the maximum dosage of 640 g/ml.

Moreover, the anticancer activity was investigated against two types of human promyelocytic leukemia cell lines (NB4 and HL-60). Both cell lines were inhibited by MC extract, with greater death percentages against NB4 cells (87.01% at 200 g/mL) than HL-60 cells (77.02% at 200 g/mL) (Khan et al., 2023).

(d) Stress reduction and sleep improvement: Chamomile is well-known for its calming and sleep-inducing properties. Chronic stress and sleep disturbances have been associated to an raised risk of neurodegenerative disorders. By reducing stress and improving sleep quality, chamomile may indirectly support health of brain and decrease the risk of neurodegeneration (Dai et al., 2022). Further research, including well-designed clinical trials, is necessary to confirm these potential benefits and determine the appropriate dosage and long-term effects in humans.

2.6.3 Murraya koenigii

2.6.3.1 Botanical Properties: *Murraya koenigii* is generally known as curry leaf or sweet neem, and is a tropical plant local to the subcontinent of India (**Figure 2.13)**. It is highly valued for its aromatic leaves, which are widely used in various cuisines for their distinct flavour and aroma (Rehman et al., 2023). It is an insignificant to medium in size deciduous shrub or tree that can reach a height of 4-6 meters (13-20 feet). The leaves of *Murraya koenigii* are the most notable part of the plant. They are compound leaves, meaning they are composed of multiple leaflets. Each leaf typically has 11-21 glossy, dark green leaflets that are lanceolate or ovate in shape. The leaflets are approximately 2-4 cm long and emit a strong, characteristic aroma when crushed or bruised. This plant produces small, white, fragrant flowers that grow in clusters called panicles. The flowers have five petals and numerous stamens (Choudhury and Garg, 2007). The plant produces small, shiny, black or dark purple berries, which are technically drupes. *M. koenigii* is a tropical plant that thrives in warm and humid climates. The leaves of *M. koenigii* are the most utilized part of the plant. Aside from its culinary applications, curry leaf is said to have a variety of therapeutic characteristics and is utilised in classical Ayurvedic medicine for its possible medical advantages (Samanta et al., 2018).

Murraya koenigii has long been utilised in traditional medicine to treat a wide range of ailments. Among other things, it is expected to have antibacterial, anti-inflammatory, anti-cancer, and antioxidant activities (Tan et al., 2022).

2.6.3.2 Taxonomic Classification of *Murraya koenigii*

Kingdom: Plantae
Phylum: *Tracheophyta*
Class: Magnoliopsida
Order: Sapindales
Family: Rutaceae
Genus: *Murraya*
Species: *koenigii*
Scientific name: *Murraya koenigii*
Common name: Curry leaf

Figure 2.13: *Murraya koenigii*

2.6.3.3 Phytochemical Properties: *Murraya koenigii*, or curry leaf, contains several phytochemicals that contribute to its medicinal properties and health benefits. Carbazole alkaloids, such as mahanimbine, girinimbine, and mahanine, are the main bioactive elements present in *M. koenigii*. These compounds possess various biological activities, including anti-microbial, anti-inflammatory, antidiabetic, and antioxidant properties (Balakrishnan et al., 2020). It also contains flavonoids such as kaempferol, rutin, and quercetin which are recognised for their anti-inflammatory and antioxidant qualities, and they also exhibit potential anticancer properties. *Murraya koenigii* is a rich source of triterpenoids, including beta-sitosterol, stigmasterol, and lupeol which have been linked with various health benefits, such as anti-inflammatory, antimicrobial, antidiabetic, and hepatoprotective properties. Coumarins, such as scopoletin and isoscopoletin possess antioxidant, anti-inflammatory, and antimicrobial properties. *M. koenigii* leaves also contain essential oils, which contribute to its distinct aroma and flavour. The essential oils are composed of various compounds, including beta-caryophyllene, alpha-pinene, and beta-pinene, which have antimicrobial and insecticidal properties (Gupta et al., 2011).

These phytochemicals present in *Murraya koenigii* contribute to its medicinal properties and make it a valuable plant in traditional medicine and herbal remedies (Utaipan et al., 2017).

2.6.3.4 *In-vivo* Pharmacological studies on *Murraya koenigii*

Murraya koenigii, or curry leaf, possesses various pharmacological properties that have been studied and documented. Some of the notable pharmacological properties of *M. koenigii* include:

(a) Anti-cancer activity: Some studies have investigated the potential anti-cancer properties of *Murraya koenigii*. In HepG2 cells, carbazole extracted from *M. koenigii* bark produce considerable targeted cell death (Syam et al., 2011). Bhattacharya et al. (2010) suggested the engagement of death receptor determined extrinsic mechanism of apoptosis by mahanine. It inhibited tumour growth in MOLT-3 cells though not in K562 cells (Bhattacharya et al., 2010). Pyrayafoline, murrafoline, and three carbazole alkaloids were also shown to be effective against HL-60 cells. Amna et al. revealed the potential of leaves by demonstrating that they are cytotoxic against HeLA cancer cell lines (Amna et al., 2019).

(b) Antioxidant activity: *Murraya koenigii* exhibits significant antioxidant properties due to the existence of several secondary metabolites including alkaloids, flavonoids, and phenolic constituents.

According to the research, aqueous extracts of *M. koenigii* leaves shown a strong protective mechanism against cadmium-induced damage to the cardiac tissues of rats. In mice, the benzene component of *Murraya koenigii* was demonstrated to have antioxidant and antimutagenic activities. These antioxidants aid in the neutralisation of radicals that are free and the protection of cells from oxidative damage, which may lessen the likelihood of chronic illnesses such as cancer and cardiovascular disease (Zahin et al., 2013).

(c) Antipyretic activity: *Murraya koenigii* ethanolic extract was found to have considerable antipyretic efficacy in a yeast-induced pyrexia rat model. Rageeb et al. tested the antipyretic efficacy of *M. koenigii* leaves on albino rats using a yeast-induced pyrexia paradigm. The findings were equivalent to the commercial antipyretic, paracetamol. In rats, an alcohol extract of *M. koenigii* demonstrated a substantial antipyretic effect in PGE1-induced hyperpyrexia (Malode et al., 2021).

(d) Immunomodulation activity: Methanolic extract obtained from *M. koenigii* leaves increased the phagocytic index significantly, owing to the fast elimination of carbon particles from the circulation. There was also an increase in antibody against ovalbumin and protection against cyclophosphamide-induced myelosuppression. MK possess immunomodulatory qualities that enhanced humoral immunity and phagocytic activity (Shah et al., 2008).

(e) Hypoglycaemic activity: Studies have indicated that *Murraya koenigii* may have antidiabetic properties. Recent report demonstrated that methanolic and aqueous extracts of *M. koenigii* leaves lowered plasma glucose levels in alloxan-induced rats. The ethanolic extract of MK stem demonstrated significant decrease in triglyceride, blood glucose level, body weight, and total cholesterol. Mahanimbine, derived from the leaves of *M. koenigii*, was discovered to have hypolipidemic and antihyperglycemic properties (Husna et al., 2018).

(f) Anti-ulcer activity: The anti-ulcer activity of hot aqueous fraction of leaves was studied at two different dosages of 250 and 400 mg/kg. The fraction prevented stomach lesions caused by anti-inflammatory medications and a pylorus ligation model. In the pylorus ligation model, the fraction reduced ulcerative lesion and stomach volume while increasing the pH of gastric juice (Mani et al., 2013).

While *Murraya koenigii* has shown these pharmacological effects in several investigations, additional study is needed to properly examine its potential medicinal uses.

2.7 OBSTACLES OF DRUG DELIVERY TO BRAIN TARGETING

Delivering medicines and nutritional antioxidants to particular brain sites is complicated by the existence of the blood-cerebrospinal fluid barrier (BCFB), blood-brain barrier (BBB), and other physiological variables (Gosselet et al., 2021). Despite the screening of several pharmacological candidates, only a few options, such as tacrine, donepezil, galantamine (AChE inhibitors), galantamine, rivastigmine, curcumin, and memantine (NMDA inhibitor), are now used in the therapeutic treatment of neurological disorders. The CNS is probably the most complicated and sensitive system in the human body. Because of the complicated architecture of the CNS, there are several restrictions in targeted medicine transfer from the blood brain to the CNS. These include a lack of knowledge regarding the pharmacokinetics (half-life), bioavailability for brain cells, or drug's function, as well as adverse effects and the unexpected interaction of off-targets-drugs with unspecific enzymes and receptors (Goyal et al., 2014). This is a consequence of the convoluted pharmacology of various drugs, the extended idleness time of neurodegenerative problems, the ineffectualness of prescriptions after the movement of sicknesses, erroneous measurements, the genotype of the patient populace and differing reactions to drugs, and the instability (oxidation, hydrolysis) record of assessed drugs. In addition, the issues will only become worse in terms of the brain (Huttunen et al., 2022). The three primary obstacles to medication delivery into the brain will be described here.

2.7.1 Blood-brain barrier (BBB)

The BBB is a semi-permeable and highly selective barrier that separates circulating blood from the CNS, which includes the spinal cord and brain. Its primary role is to protect the brain from potentially harmful substances and maintain a stable environment necessary for proper neuronal function. It is comprised of particular endothelial cells that coat the walls of vessels in the mind (Ronaldson and Davis, 2011). These endothelial cells are tightly connected by junctional complexes, including tight junctions, which limit the passage of molecules and ions between cells. The tight junctions restrict the movement of most substances, including large molecules and many drugs, from freely diffusing into the brain. Aside from endothelial cells, the BBB is maintained by additional cell types such as astrocytes, pericytes, and the basal lamina. This prevents the free movement of substances between cells and limits the diffusion of hydrophilic molecules (Ding, et al., 2020). P-glycoprotein and other specialised transport proteins are found on the lumen (blood-facing) surface of cell membranes. These transporters actively pump certain drugs and toxins out of the brain, reducing their accumulation. The BBB

contains enzymes that can metabolize certain substances, further limiting their ability to enter the brain. Astrocytes, a type of glial cell, have projections called endfeet that surround the blood vessels. They help to regulate the passage of waste products and nutrients among the circulatory system and the brain (Patel et al., 2017).

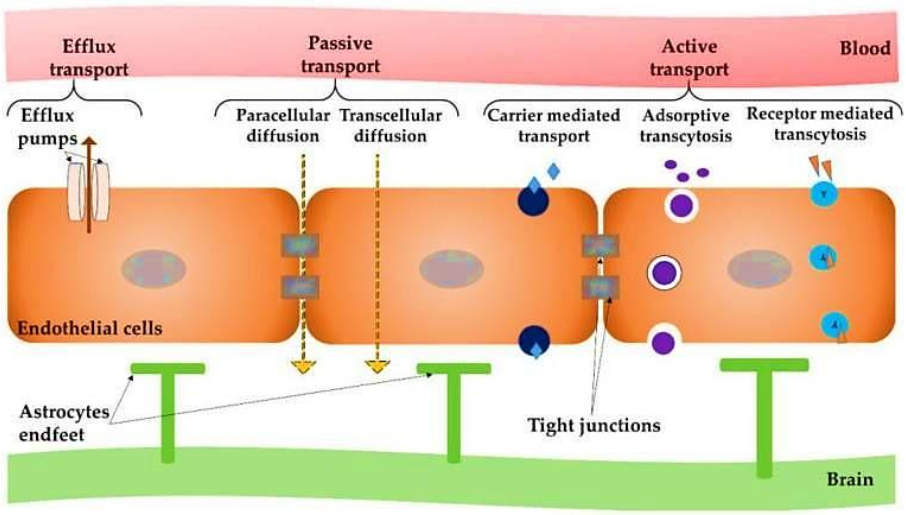


Figure 2.14: Multiple routes of transport through the blood-brain barrier (BBB) (Teleanu et al., 2019).

Surrounding brain capillary endothelial cells are microglial cells, astrocytes, pericytes, and extracellular matrix. The basal lamina, which is made up of fibronectin, laminin, heparin sulphate, and type IV collagen, surrounds the brain base endothelial cells (**Figure 2.14**). However, over 97.9% of drugs used to treat brain illnesses do not cross the BBB, leaving these exceedingly complex conditions untreated (Xie et al., 2019).

Although the BBB is important for preserving the functioning of the CNS, it also makes medicine transport to the brain difficult. Many therapeutic agents, including drugs and dietary antioxidants, face difficulties in crossing the BBB and reaching their target sites within the brain (Zhang et al., 2016). Researchers are actively investigating various strategies to overcome these challenges and develop methods for targeted drug delivery across the BBB.

2.7.2 Blood-cerebrospinal fluid barrier (BCSFB)

BCSFB is a specialised barrier system that controls the flow of chemicals inside the central nervous system between blood and cerebrospinal fluid . It is one of numerous barriers that keep potentially dangerous compounds out of the spinal cord and brain. The BCSFB is primarily produced by the choroid plexus, a layer of specialised cells located in the brain's ventricles (Redzic, 2011). The choroid plexus is made up of an arrangement of blood arteries surrounded by cells of epithelium that coat the ventricles. Tight junctions link these cells,

forming a physical barrier that inhibits the passage of chemicals among the CSF and blood (Engelhardt and Sorokin, 2009).

BCSFB poses significant obstacles to drug delivery for brain targeting as sown in **Figure 2.15**. While BBB is the primary barrier that restricts the passage of substances into the brain tissue, the BCSFB is also involved in preventing drug access into the CSF and, ultimately, the brain. Tight associations connect the cells of the epithelial layer of the choroid plexus, which comprise the BCSFB. These tight junctions create a physical barrier that restricts the paracellular movement of molecules (Strazielle and Ghersi, 2016). Similar to the BBB, the BCSFB is equipped with efflux transporters, including P-gp, which actively pump drugs out of the CSF and back into the blood. These transporters significantly limit the accumulation of drugs in the CSF and brain. Even if a drug manages to cross the BCSFB, it may still face challenges in permeating brain tissue due to the presence of the BBB (Ueno et al., 2016). The drug needs to cross both the BCSFB and the BBB to reach its target site within the brain, further complicating drug delivery. The permeability of the BCSFB can vary depending on the specific region of the brain and the drug being delivered. Some areas of the brain may have a more restrictive BCSFB compared to others, making drug delivery inconsistent. Unlike the BBB, which has specific transport systems for certain molecules, the BCSFB has limited specific transport mechanisms (Wang and Zuo, 2018). Because of the lack of specialised transporters, targeted medication delivery to the CSF and brain might be difficult.

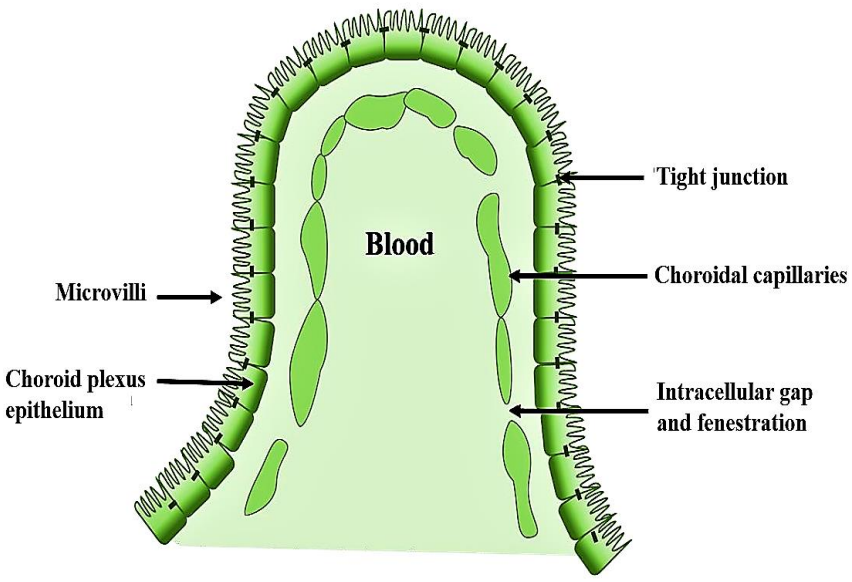


Figure 2.15: A schematic representation of the Blood-Cerebrospinal Fluid Barrier.

Overall, the BCSFB's limitations in delivering medicines to the brain underscore the need for novel techniques and focused ways to successfully transport medications to the CSF and brain tissue during the treatment of a variety of neurodegenerative illnesses.

2.7.3 Multidrug resistance protein 1 (MDR1)

P-glycoprotein (P-gp), also known as multidrug resistance protein 1 (MDR1), is a membrane transporter protein that serves a crucial impact in protecting tissues, including brain, from potentially harmful substances by actively pumping them out of the cells. While P-gp serves as a defense mechanism, it can also present significant obstacles to drug delivery to the brain (VanDuyn and Nass, 2014). P-gp is abundant in the BBB, a specialized structure that strictly restricts the movement of chemicals from the circulation into the brain. The presence of P-gp reduces the permeability of drugs, making it difficult for them to cross the BBB and reach their intended targets in the brain as illustrated in **Figure 2.16**. P-gp actively transports a wide range of drugs and other compounds out of brain cells back into the bloodstream (Dallas et al., 2016).

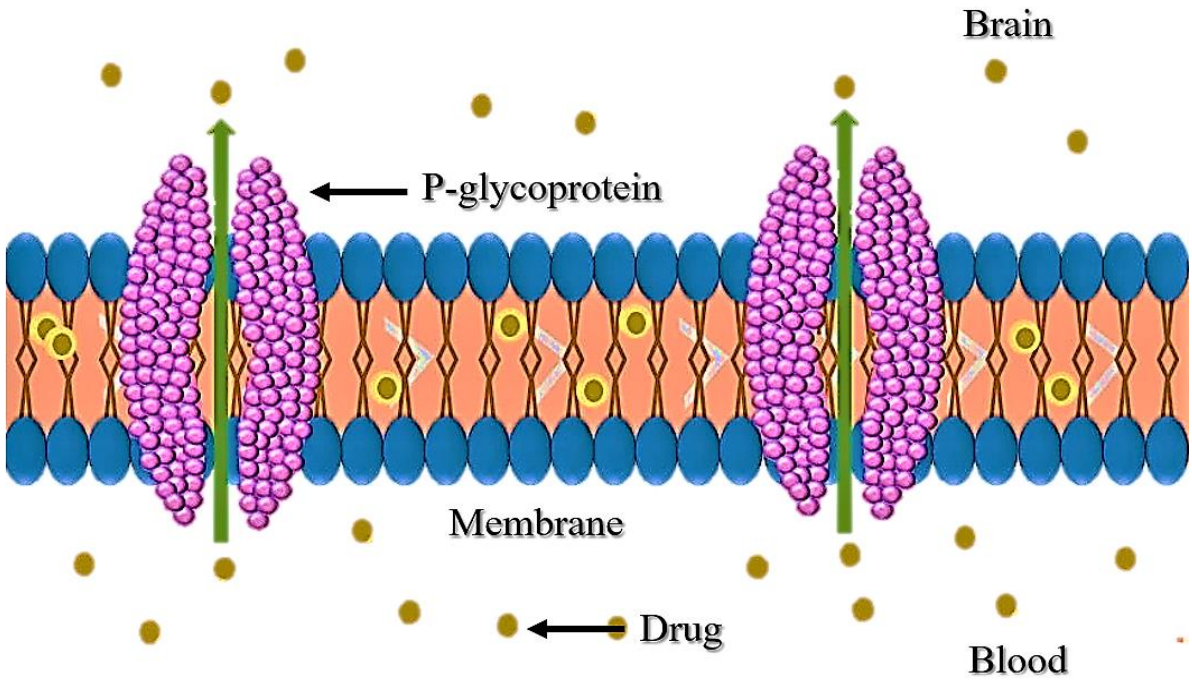


Figure 2.16: The structure of P-glycoprotein in cerebral endothelial cells is shown schematically.

This efflux action can significantly reduce the concentration of drugs in the brain, limiting their therapeutic efficacy. P-gp effectively acts as a barrier, preventing drugs from accumulating in sufficient amounts in the brain (Amin et al., 2013). Overexpression of P-gp can lead to drug resistance in certain diseases, such as cancer. P-gp pumps out chemotherapeutic drugs from cancer cells, reducing their effectiveness (Cole, 2014). This resistance can also extend to drugs targeting brain disorders like epilepsy and neurodegenerative diseases, further complicating treatment. P-gp can interact with multiple drugs simultaneously due to its broad substrate

specificity (Gupta et al., 2015). When multiple drugs are administered together, they can compete for binding and transport by P-gp, potentially affecting the pharmacokinetics and bioavailability of each drug. This can lead to unpredictable drug interactions and altered therapeutic outcomes (Grant et al., 2008). This variation adds another degree of complication to medication transport to the brain, since therapeutic efficacy may vary based on P-gp expression levels (Rathod et al., 2022). Overcoming these P-gp-related medication transport barriers is an ongoing topic of study. Strategies including developing P-gp inhibitors, prodrugs, nanocarriers, and targeted delivery systems are being explored to enhance drug permeability across the BBB and circumvent P-gp-mediated efflux (Haimeur et al., 2002).

The existence of the blood-cerebrospinal fluid barrier (BCFB), blood-brain barrier (BBB), and efflux transporters such as P-glycoprotein (P-gp) makes delivering medicines and antioxidants to particular sites in the brain difficult (Masoudi et al., 2020). Nanotechnology provides a versatile platform for designing and optimizing drug delivery systems to overcome the barriers in the brain (Parhi et al., 2012). By utilizing nanocarriers, surface modifications, targeting ligands, and other strategies, it becomes possible to enhance drug penetration, protect therapeutic agents from degradation, and increase their accumulation at the desired sites within the brain (Leslie et al., 2001).

2.8 NANOTECHNOLOGY

Nanotechnology is a novel and fast evolving technique with several potential applications. Nanoparticles are tiny particles typically ranging from 1 to 100 nanometres (Pardhi et al., 2018). In recent years, nanomaterials including nanotubes, nanofibers, nanoparticles, and quantum dots have found widespread use in biomedical applications including drug delivery, biological imaging, and biosensors (Boote et al., 2014). Nanoparticles can be used to encapsulate and deliver therapeutic drugs to specific target sites in the body, improving drug efficacy and reducing side effects (Sana et al., 2021).

Nanoparticles can be engineered to carry therapeutic agents, such as drugs or biomolecules, to specific locations in the brain. These nanoparticles can cross the BBB, a protective barrier that prevents many substances from entering the brain, and deliver the drugs directly to the affected areas (Gour and Jain, 2019). Nanoparticles can be designed to provide neuroprotective effects. Additionally, nanoparticles can be used to deliver growth factors or other molecules that promote neuronal survival and regeneration, potentially slowing down disease progression (Ying et al., 2022).

2.9 GREEN SYNTHESIS OF NANOPARTICLES

The generation of nanoparticles using ecologically friendly and sustainable technologies is referred to as green synthesis of nanoparticles. These approaches attempt to limit or eliminate the use of harmful chemicals and potentially dangerous procedures that are commonly connected with traditional nanoparticle manufacturing (Hussain et al., 2016). Green synthesis offers several advantages, including lower environmental impact, cost-effectiveness, and potential biocompatibility for biomedical applications. Some common approaches for the green fabrication of nanoparticles are plant fractions, microorganisms (fungi, and algae), bio-waste Materials (agricultural waste, food waste, and other bio-waste materials), solar irradiation, and green solvents (Qiao et al., 2022). Plant extracts, such as leaves, stems, roots, or fruits, are commonly used in green synthesis. These fractions contain natural chemicals such flavonoids, polyphenols, terpenoids, and proteins that can function as stabilising agents in the creation of NPs. The extract is mixed with metal precursors, and the reduction reaction occurs under mild conditions, leading to the formation of nanoparticles. Various plants, such as *Aloe barbadensis* miller, *Camellia sinensis*, *Azadirachta indica*, and *Curcuma longa*, have been used for nanoparticle synthesis (Jadoun et al., 2021).

2..9.1 Mechanism of plant-based green synthesis of nanoparticles

The selected plant material is thoroughly washed, dried, and finely ground. The ground plant material is then mixed with a suitable solvent, such as water, ethanol, or a combination of both, to obtain a plant extract (Hano and Abbasi, 2021). The plant fraction involves a variety of biologically active substances that function as reducing and stabilizing agents, including phenolics, flavonoids, alkaloids, and terpenoids. These compounds can effectively reduce metal ions present in a solution (usually metal salts) to their corresponding metallic form. The reduction process involves the transfer of electrons from the bioactive compounds to the metal ions, leading to their reduction and subsequent formation of nanoparticles (Sood and Chopra, 2018). Once the reduction of metal ions occurs, nucleation takes place, where small clusters of metal atoms are formed. These clusters act as seeds for further growth and aggregation. X-ray diffraction (XRD), UV-Vis spectroscopy, TEM, and FTIR are used to determine the size, shape, crystallinity, and chemical composition of the synthesised nanoparticles.

The nanoparticles can then be applied in various fields, including medicine, catalysis, agriculture, and environmental remediation (Anand et al., 2007).

The specific mechanisms involved in plant-based green synthesis can vary depending on the plant material, the bioactive compounds present, and the metal ions being used. Additionally, the optimization of synthesis parameters such as pH, temperature, and reaction time can also influence the synthesis process and the properties of the resulting nanoparticles (Andra et al., 2019).

2.9.2 Different types of plant-derived nanoparticles

Plant-derived NPs can be synthesized using various plant materials and their extracts. The type of plant material and the bioactive compounds present determine the properties and applications of the resulting nanoparticles (Aljabali et al., 2022). The plant fraction involves a variety of biologically active substances that function as reducing and stabilizing agents. These compounds can effectively reduce metal ions present in a solution to their corresponding metallic form. Here are some examples of plant-derived nanoparticles:

(a) Silver nanoparticles (AgNPs): Silver nanoparticles fabricated from plant fractions have gained significant attention due to their antimicrobial properties. Plants such as *Aloe barbadensis* miller, *Camellia sinensis*, *Azadirachta indica*, and *Allium cepa* have been used for the green fabrication of AgNPs (Alharbi et al., 2022).

(b) Gold nanoparticles (AuNPs): Gold nanoparticles derived from plant extracts exhibit unique optical properties and have applications in biomedicine, catalysis, and sensing. Plants such as *Curcuma longa*, *Camellia sinensis*, *Vitis vinifera*, and *Rosa rubiginosa* have been utilized for the fabrication of AuNPs (Ahmed et al., 2016).

(c) Iron nanoparticles (FeNPs): Iron nanoparticles derived from plant extracts find applications in environmental remediation, water treatment, and magnetic devices. Plants such as *Eucalyptus*, *Azadirachta indica*, and *Terminalia chebula* have been used for the fabrication of FeNPs (Ebrahiminezhad et al., 2018).

(d) Copper nanoparticles (CuNPs): Copper nanoparticles synthesized from plant extracts possess excellent antimicrobial, catalytic, and electrical conductivity properties. Plants such as *Allium sativum*, *Camellia sinensis*, and *Vitis vinifera* have been utilized for the green synthesis of CuNPs (Thiruvengadam et al., 2019).

(e) Titanium dioxide nanoparticles (TiO_2NPs): TiO_2NPs are widely used in photocatalysis, solar cells, and environmental applications. Plant extracts from plants like *Aloe barbadensis* miller, *Camellia sinensis*, and *Citrus limon* have been employed for the synthesis of TiO_2NPs (Sunny et al., 2022).

2.10 ZINC OXIDE NANOPARTICLES (ZnO-NPs)

Zinc oxide (ZnO) is an II-VI semiconductor. It is an impermeable in water white solid powder (Król et al., 2017). Although zinc oxide occurs organically as the mineral Zincate, it is typically synthesized. Its crystal formations include cubic zinc mixture and hexagonal wurtzite (Jiang et al. 2018). Because of the presence of oxygen deficit and/or zinc interstitials, ZnO is an inherently n-type semiconductor (**Figure 2.17)**. ZnO is widely recognised for its many features, including pyroelectricity, piezoelectricity, and semi conductivity (Fouda et al., 2018). It also has a relatively broad band gap and a significant excitonic binding energy of 60 meV, allowing it to be used in a variety of optoelectronic devices (Kavitha et al. 2023).

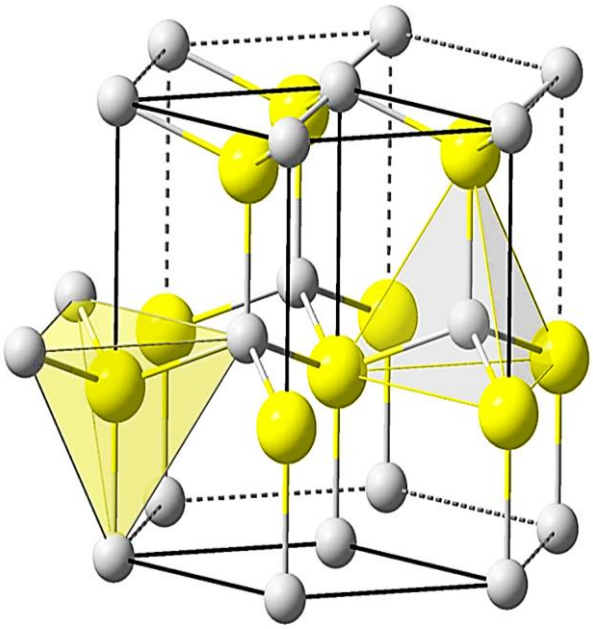


Figure 2.17: The tetrahedral coordination is depicted for both kinds of atoms in the ZnO wurtzite cell, with Zn in yellow and O in grey.

ZnO nanoparticles (ZnO-NPs) are zinc and oxygen atom nanoparticles. Because of their tiny size and elevated surface area to volume ratio, they have particular physical, chemical, and optical characteristics. ZnO-NPs have grabbed the interest of numerous sectors due to their unique properties and potential applications (Manojkumar et al., 2023).

2.10.1 Literature Survey on plant-derived ZnO-NPs

A literature survey on plant-derived ZnO-NPs reveals a growing interest in utilizing natural sources to synthesize nanoparticles with various applications. Because of their rich phytochemical makeup, plant extracts provide a green and sustainable method to the production of nanoparticles, including ZnO-NPs (Kader et al., 2023).

Ambujakshi et al. (2019) have found the anti-cancer activity of biosynthesized ZnO-NPs from *Chonemorpha grandiflora* extract, which demonstrated notable toxicity results. A-549, MCF-7, and HCT-116 cell lines were used to investigate the *in vitro* cytotoxic properties of zinc-oxide NPs. The phyto-synthesized ZnO-NPs had varying harmful effects on several cell lines; however, cell line toxicity is dependent on nanoparticle dose. The practicability tests additionally show that rising the quantity of nanoparticles extensively diminishes cell

manageability; likewise, cell maintainability that kicked the bucket in the accompanying grouping was noted: MCF-7>HCT-116>A-549. Wahba et al. (2016) exhibited that ZnO NPs effectively corrected diabetes-incited pancreatic harm, as confirmed by underlying and ultrastructural improvement and biochemical standardization of serum insulin and blood glucose levels. Ali et al. (2016) green manufactured ZnO-NPs using Aloe barbadensis miller leaf broth fraction. When compared to chemically synthesised ZnO-NPs, their results demonstrated greater biocidal potential against a variety of illnesses. They also discovered that raising the particle dose, treatment time, and manufacturing procedure improved nanoparticle efficiency. According to a recent study, NPs containing zinc-II cation generate reactive oxygen species in cancerous cells, finally eliminating them. Disease cells die on because of zinc oxide-based nanoparticles modifying histone methylation and prompting modified cell demise in them by making countless free extremists. Plant-based zinc oxide nanoparticles create higher hurtful impacts against HepG-2 cell line than ordinary cells like NIH-3T3, which was time and sum subordinate (Ehsan et al., 2022). Nagajyothi et al. (2021) detailed that ZnO-NPs were tried for cytotoxicity in ovarian (SKOV-3) and cervical (HeLa) malignant growth cell lines. The portion subordinate cytotoxicity was inspected by estimating cell reasonability, ROS creation, and adenosine triphosphate (ATP) levels following 48 hours of openness to ZnO NPs. The 2.0 mg/mL ZnO-NPs showed higher anticancer adequacy against SKOV-3 and HeLa cell lines among the different portions of ZnO-NPs produced. Cell feasibility and ATP content were extensively diminished in a portion subordinate way, while ROS creation was essentially supported. Lipovsky et al. (2011) found a focus subordinate effect of ZnO-NPs on Candida albicans suitability. ZnO-NPs mind grouping of 0.1 mg/ml diminished the reasonability of C. albicans by over 95%. Energizing ZnO-NPs with apparent light helped yeast cell demise much more. In light of areas of strength for its capacities and epithelialization-animating effect of zinc, ZnO-NPs have likewise been utilized effectively in injury dressings (Lansdown et al., 2007).

Plant-determined ZnO-NPs have shown mitigating properties by repressing favorable to incendiary arbiters and cytokines, for example, IL-6, growth putrefaction factor-alpha (TNF-α), and nitric oxide (NO), Plant-determined ZnO-NPs have been examined for their true capacity in treating diabetes, cardiovascular sicknesses, and skin problems (skin break out) however very little data is accessible to treat neurodegenerative illnesses including AD (Sumanth et al., 2020).

2.11 NANOPARTICLES FOR THE TREATMENT OF NEUROLOGICAL DISORDERS

Engineered nanoparticles developed as medications for neurological diseases are referred to as neuronanomedicine. Nanotechnology creates nanoscale devices by engineering nanomaterials that communicate with biological processes at the level of the molecular (Ashraf et al., 2018). These nanotechnological products can be utilised to activate, respond to, and connect to target cells and tissues to accomplish an intended physiological response while minimising side effects. Some NPs have been designed to cross the BBB and target specific areas within cells (Barbu et al., 2009). Their research focuses on finding extracellular or intracellular molecules, such as amyloid beta plaques in AD. Surface alteration of nanoparticles to make exceptionally certain particles would permit them to cross the BBB through adsorptive and receptor/carrier intervened transcytosis as depicted in **Figure 2.18** (Migliore et al., 2015).

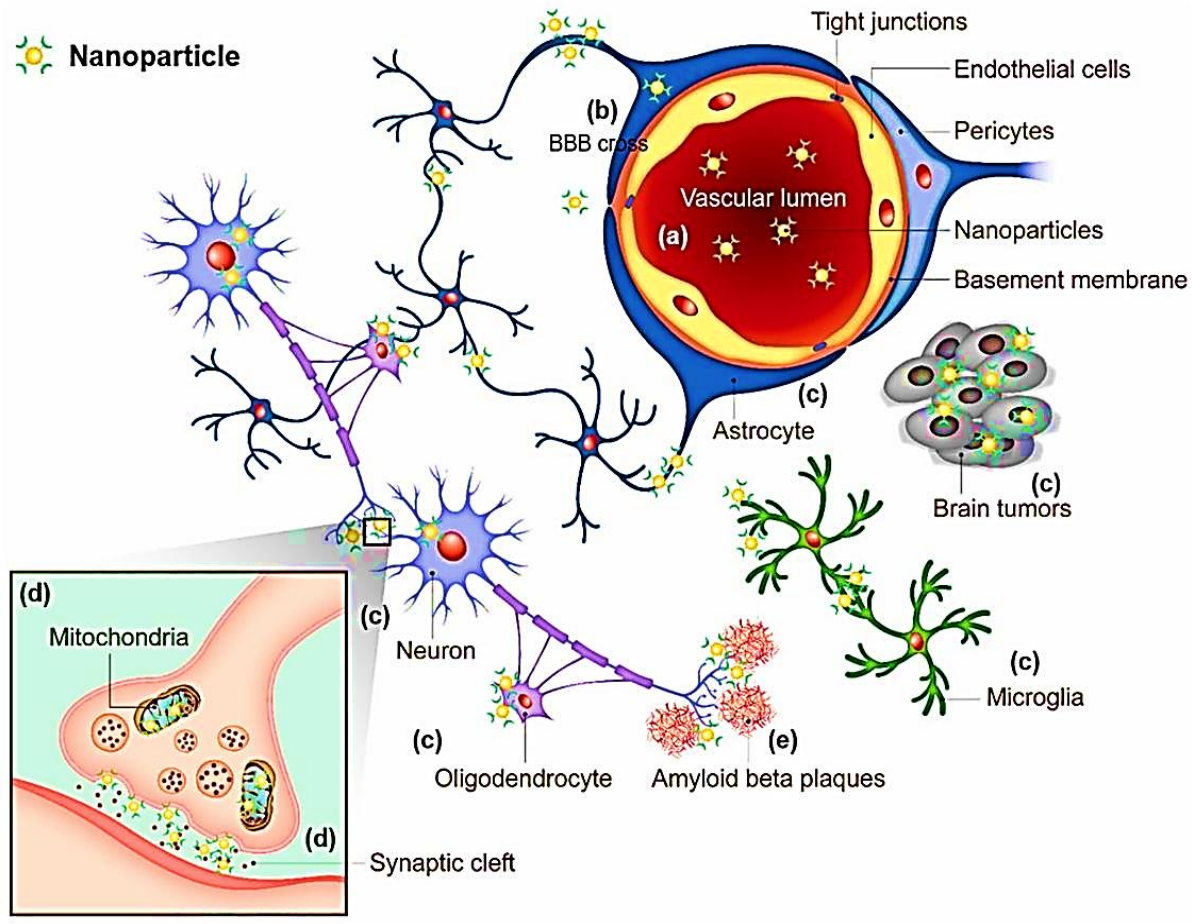


Figure 2.18: Nanoparticles with focusing on properties are being utilized to treat mind infections. (a) NPs flowing in the circulation system, (b) should cross the BBB, (c) then restrict to target cells (neurons, astrocytes, oligodendrocytes, growth cells, microglia), and (d) once in a while target cell organelle (synaptic split, mitochondria), or (e) extracellular particles (amyloid beta plaques in Alzheimer's illness) (Jin et al., 2020).

The recognition of apolipoprotein E by particular BBB receptors increases lipoprotein transport to the brain. Apo E conjugation to albumin-NPs or liposomes, for example, might enable NPs to be detected and transmitted by particular BBB receptors. (Hawwary et al., 2022).

The mechanism of plant-derived nanoparticles (NPs) for the therapy of neurological diseases involves several pathways and interactions within the CNS. Plant-derived NPs, such as ZnO NPs, often possess inherent antioxidant properties due to the existence of secondary metabolites (Heenatigala et al., 2019). These nanoparticles can scavenge free radicals and ROS in the brain, lowering oxidative damage, which has been linked to neurodegeneration. Plant-derived nanoparticles may help prevent or reduce the course of neurodegenerative disorders by shielding neurons from oxidative damage (Nazıroğlu et al., 2017). Plant-derived NPs exhibit anti-inflammatory effects, suppressing the generation of pro-inflammatory cytokines such as interleukin-1 and TNF-α. These NPs may ameliorate neuronal injury and neurodegeneration by lowering neurological inflammation (Jha et al., 2023). Neuroprotective effects of phyto-engineered NPs may be achieved through increasing the survival of neurons and stimulating neuronal development and diversification. These NPs have the ability to boost neurotrophic factors including BDNF. which are important for neuronal maintenance and development (Thukral et al., 2023).

Green nanoparticles can also have an effect on neurological protection and the survival of cells signalling pathways including the PI3K/Akt and ERK/MAPK pathways. Some neurodegenerative disorders, such as AD, involve the abnormal accumulation of metal ions (e.g., copper, zinc) and protein aggregates (e.g., amyloid-beta) in the brain. Plant-derived NPs, including ZnO-NPs, can act as metal chelators, binding to metal ions and preventing their harmful interactions. Furthermore, these NPs may suppress amyloid-beta protein aggregation, lowering the development of harmful amyloid plaques associated to dementia (Sohail et al., 2020).

Plant-derived nanoparticles have the potential to be soothing by reducing pro-inflammatory chemicals such as cytokines and enzymes such as inducible COX-2 and iNOS. They have the potential to alter the immune response and decrease damage in the brain. They have the ability to boost neurotrophic factors like BDNF and NGF, which support neurological development and function. Furthermore, these nanoparticles may promote synaptogenesis (the development of new synapses) and neurogenesis (the growth of new neurons), both of which are necessary for neural repair and recovery. Metal ion abnormal build-up, including iron, zinc, and copper

has been reported in neurodegenerative illnesses and leads to neuronal damage (Ullah et al., 2022). Certain plant-based nanoparticles have metal chelating properties, which means they can bind to these metal ions and help maintain metal ion homeostasis in the brain (Nobahar et al., 2021). Plant-synthesized NPs can be engineered to possess properties that facilitate their transport across the BBB. Once in the brain, these NPs can deliver therapeutic agents, such as antioxidants, anti-inflammatory compounds, or neuroprotective drugs, directly to the affected regions (Shanmuganathan et al., 2019). The targeted delivery of drugs using green NPs may enhance their effectiveness while minimizing systemic side effects (Akintunde et al., 2021).

It is important to note that the precise processes of plant-derived NPs in the treatment of neurodegenerative diseases may differ depending on the NP formulation, plant source, and disease model. More research is required to fully comprehend these pathways and maximise the use of plant-derived NPs for effective and safe neurodegenerative disease treatment.

2.12 DRUGS IN CLINICAL TRIALS

There are currently no effective or disease-modifying drugs for neurodegeneration. Amyloid beta aggregation, alpha synuclein accumulation, tau accumulation, neuroinflammation, cognitive decline, and the beginning of psychiatric and behavioural disorders are all biochemical and clinical phenomena that occur as neurodegeneration proceeds (Andrade et al., 2023). Clinical studies to prevent these incidences are now being examined. Nutraceuticals are also being studied in clinical studies (Stanzione and Tropepi, 2011).

Since anti-amyloid studies have failed in recent years, researchers' focus has shifted to those who have positive diagnostic biomarkers at the prodromal or preclinical stage. Meanwhile, the amyloid theory has been called into doubt, and the number of anti-amyloid phase 3 studies has dropped in 2019. Phase 1 and phase 2 trials include a wide variety of aims, with a growing emphasis on neuroprotection and anti-neuroinflammation in phase 1 and phase 2 trials, respectively. Two or more drugs are typically required to successfully arrest the course of chronic progressive disorders (Chopade et al., 2022). Clinical trials are a dynamic process, and the success or failure of a drug in trials can influence its progression and availability for clinical use (Puopolo and Pocchiari, 2011).

Anti-Aβ monoclonal antibodies and other medications, including nutraceuticals, are being tested in clinical studies, as mentioned in **Table 2.3.**

Table 2.3: Drugs in clinical trials.

S.No.	Investigational drug/nutraceutical(Category)	Mechanism of action	Sponsor (Clinical trial end date)/References
1.	Gantenerumab (MA)	Remove amyloid plaque in the brain	Roche (November 2019)
2.	Cilostazol (Vasodilator)	PDE3 antagonist	National Cerebral & CardiovascularCenter, Japan (December 2020)
3.	Aducanumab [Monoclonal antibody(MA)]	Remove amyloid plaque	Biogen (April 2022)
4.	Deferipirone (Iron chelator)	Reduces reactive oxygen species (ROS) that can harm neurons, making it neuroprotective.	Neuroscience trials, Australia (December 2021)
5.	CNP520 (Amyloid vaccine)	Reduces amyloid formation by inhibiting APP site cleavage.	Axsome therapeutics (July 2024)
6.	Telmisartan (Angiotensin receptor blocker)	Improve vascular functioning	Sunnybrook Health Sciences Centre(March 2021)
7.	Crenezumab (MA)	Remove amyloid plaque	Roche/Genentech (July 2021)
8.	Methylphenidate (Neurotransmitterbased)	Improves clinical symptoms by inhibiting dopa-mine re-uptake.	Johns Hopkins (August 2020)
9.	Solanezumab (MA)	Remove amyloid plaque	Eli Lilly (July 2022)
10.	Grape Seed Extract (Nutraceutical)	Anti-Oligomerization Agent	Mount Sinai AD Research Center(September 2020)
11.	Icosapent ethyl (purified form of Omega3 fatty acid EPA (Omega 3 FA)	Neuroprotective, affords protection from disease pathology	University of Wisconsin (November2021)
12.	Dronabinol (CB1 and CB2 endocanna-binoids partial agonist)	Improve agitation (neuropsychiatric symptoms inAD)	John Hopkins University (December2020)

2.13 *DROSOPHILA MELANOGASTER* AS A MODEL ORGANISM

Drosophila melanogaster, frequently referred to as the fruit fly, is a tiny insect in the *Drosophilidae* family. It is one of the most intensively researched creatures in biological study, notably in genetics and developmental biology. Adult *Drosophila* flies are about 3-4 mm long and have a tan or light brown body with red eyes. They have a relatively short lifespan of about 2-3 months. Under optimal conditions, they can complete their life cycle from egg to adult in about 10-14 days (Ong et al., 2015).

Drosophila **(Figure 2.19)** has a relatively small genome size of approximately 180 million base pairs, with around 14,000 protein-coding genes. The fly's genetic makeup shares many similarities with that of humans, making it a useful model organism for understanding human genetics and disease. One of the most notable breakthroughs was the identification of chromosomes as carriers of genetic information by Thomas Hunt Morgan in the early 1910s. This work laid the foundation for the field of genetics. The use of *D. melanogaster* in research has raised questions about the ethics of animal experimentation (Jeibmann and Paulus, 2009). However, its small size, short lifespan, and lack of complex emotions and cognitive abilities make it a less controversial choice compared to using higher organisms in scientific studies. *Drosophila melanogaster* and humans may seem quite different, but they share a surprising level of genetic similarity. Although the overall genetic makeup of the two species differs significantly, there are several key aspects of genetics that are conserved between them (Moloney et al., 2010). Many genes are shared between fruit flies and humans. It is estimated that about 60% of human disease-related genes have functional counterparts in fruit flies. These include genes involved in essential cellular processes, including metabolism, signal transduction, and cell division. Fruit flies have served an important role in understanding the fundamental principles of developmental biology. Many key developmental genes and pathways discovered in *Drosophila*, such as the homeobox genes, have counterparts in humans and play similar roles in embryonic development as demonstrated in **Figure 2.20** (Tue et al., 2020).

Figure 2.19: *Drosophila melanogaster*

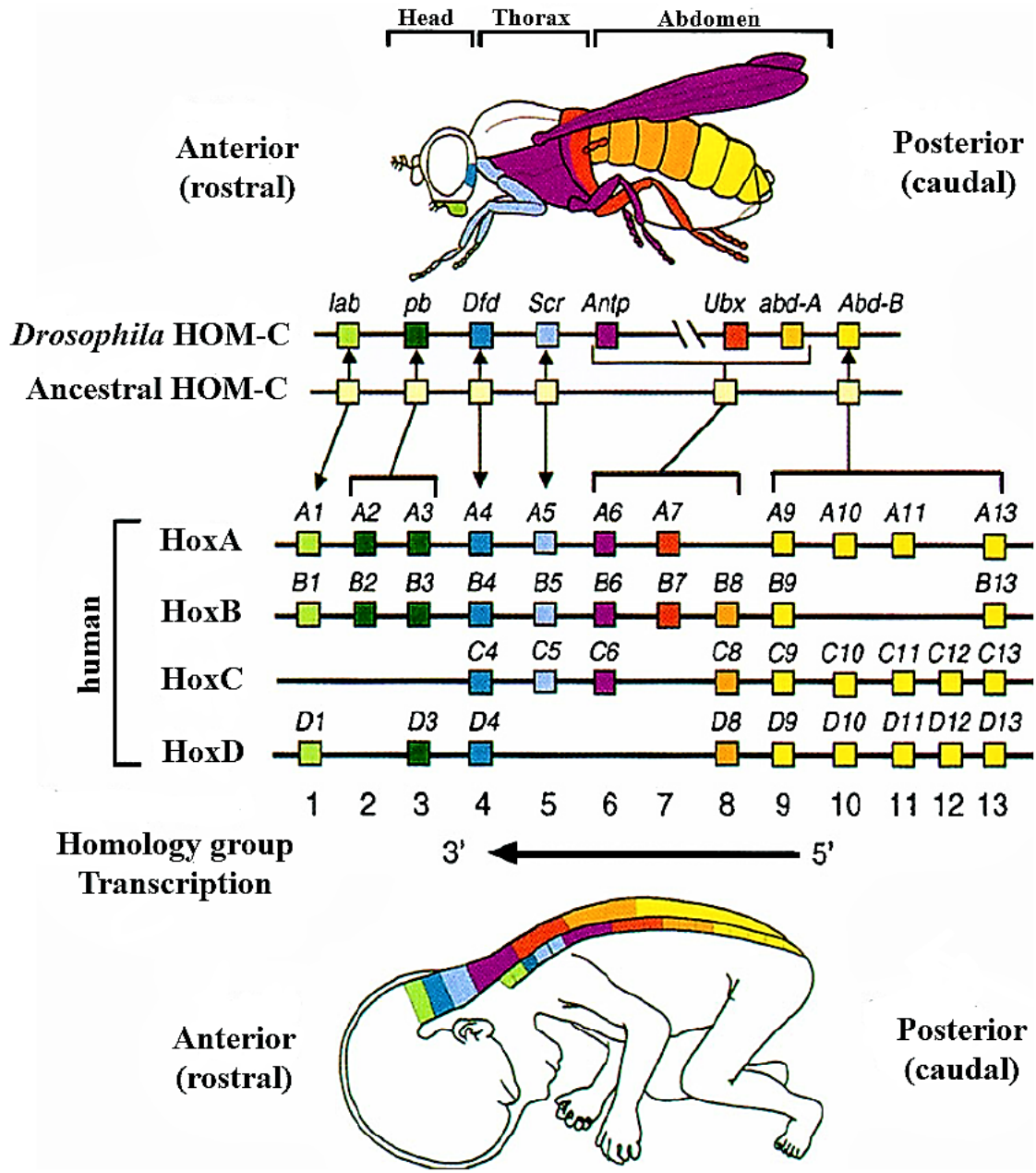


Figure 2.20: *Drosophila* HOM genes and human Hox genes have similar genomic organisation and colinear expression patterns. The *Drosophila* homeotic complex (HOM-C), the four human Hox edifices, and a potential familial homeotic complex are shown schematically, alongside their plausible phylogenic associations. A hued box addresses every gene. HOM/Hox gene articulation spaces are schematized in a fly and in the CNS and prevertebrae of a human baby (extrapolated from mouse information). To forestall bewilderment, the fractional cross-over of HOM quality records in the fly's thoracic and stomach fragments, as well as the covering articulation spaces of mammalian Hox qualities along the body pivot, are not addressed; in this way, each tone addresses the front most articulation area of a given subfamily. Lab, labial; pb, proboscipedia; Dfd, Disfigured; Scr, Sex brushes decreased; Ubx, Ultrabithorax; Antp, Antennapedia; abd-A, stomach A; Abd-B, stomach B (Horabin, 2013).

Many key developmental genes and pathways discovered in *Drosophila*, such as the homeobox genes, have counterparts in humans and play similar roles in embryonic development. Fruit flies can be genetically manipulated to model human diseases, including neurodegenerative diseases. By introducing human disease-associated genes or mutations into fruit flies, researchers can study the effects of these genetic changes on cellular and organismal phenotypes (Tickoo and Russell, 2002). Fruit fly models have provided valuable insights into the mechanisms underlying various human diseases. Despite the genetic differences between fruit flies and humans, there is functional conservation of certain genes and biological processes. For example, the basic components and mechanisms of cellular processes like DNA replication, transcription, and translation are similar in both species (Pandey and Nichols, 2011).

2.13.1 Life Cycle of Drosophila melanogaster

Fruit flies are little and easy to control. A huge number of progenies may be easily produced for molecular, genetic, and biochemical research of human disorders (Nichols, 2006). The flies have a short generation time (10-12 days) from fertilised egg to mature adult and a life span (60-80 days) depending on culture conditions. Flies are normally cultured at 24±˚C to protect fly populations, unless exceptional circumstances exist that may affect the rate of growth (Gong et al., 2005).

Its short life cycle and ease of breeding make it a great model organism for investigating numerous biological processes and genetics. *Drosophila melanogaster* has four life stages: egg, larva, pupa, and adult (Hales et al., 2015).

Egg Stage: The female fruit fly lays eggs, which starts the life cycle. Females can deposit hundreds of eggs in their lives. The eggs are normally tiny, white, and elongated, averaging around 0.5 mm in length. They are frequently deposited on rotting fruit or other organic debris, which provides a good habitat for the growing larvae (Villella and Hall, 2008).

Larva Stage: After around 24 hours, the eggs hatch into larvae, often known as maggots. The larvae are legless and go through three larval instar stages separated by moults. During this stage, they aggressively feed on the surrounding food, mostly the rotting fruit material on which they were set. The larval stage lasts around 4-6 days, depending on environmental circumstances (Nojima et al., 2014).

Pupa Stage: When the larval stage is finished, the third larval instar metamorphoses into the pupa stage. The larva next turns into a pupa, which is an immovable, dark-brown, oval-shaped structure. The fruit fly's tissues and organs undergo a stunning metamorphosis inside the pupa in order to mature into an adult. The pupal stage lasts around 4-6 days (Neville et al., 2014).

Adult Stage: The pupa goes through eclosion, which is the process by which the adult fly emerges from the pupal case. The newly emerging adult seems light and fragile at first, but gradually hardens and darkens as its wings and body develop. The fruit fly reaches complete maturation within a few hours, and its wings become fully functional for flight. *D. melanogaster's* adult stage lasts for around 2-3 weeks (Yamamoto, 2008).

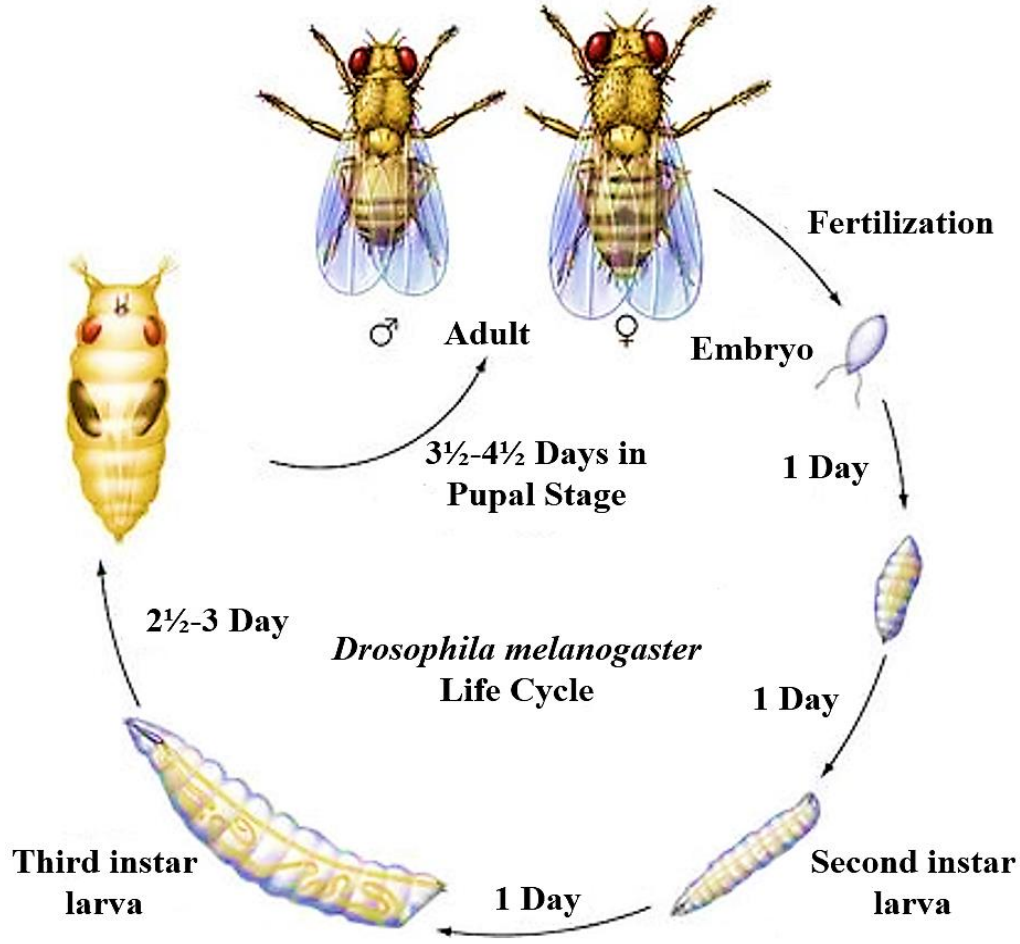


Figure 2.21: The life cycle of the fruit fly depicts the phases of development from fertilised egg to adult. Fertilized eggs hatch after one day and can live in food for up to four days as first and second instar larvae. The third instar larvae crawl outside the food (days 4-5) and pupate on the vial's edge. Metamorphosis occurs at this phase (days 6-9), and the darkening of wings begins within the pupal case, indicating that maturation is imminent. Eclosion of adult flies began around days 10-12 in pupal cases (Bjedov et al., 2010).

The fly undergoes holome-tabolous metamorphosis, which means that its life cycle is divided into four stages: fertilised egg, larva (1st instar, 2nd instar, and 3rd instar), pupa (pre, early, and late), and adult flies. Following fertilisation, the embryogenesis process took 24 hours to complete, after which moulting activities began at each larval stage change (1st, 2nd, and 3rd instar) (Helfand and Rogina, 2003). The usual time period for the first two instars larvae is one day, whereas the third instar larvae takes two days. Thus, 5 days are required following egg fertilization to complete larval development. The larvae have 19 imaginal discs that are the result of tissue-specific progenitor cells. After that, the larva metamorphoses into a pupa with a hard and protective layer of chitin and stays in the pupal case for up to 4 days. Following that, the pupa develops into an adult fly and becomes sexually mature between 8 and 12 hours. *Drosophila* prothoracic gland secretes the steroid hormone ecdysone, which is necessary for metamorphosis (Ding et al., 2022).

2.13.2 Third instar larvae of Drosophila melanogaster

The 3rd instar larvae of *D. melanogaster* are the fruit fly's penultimate larval stage before transformation into a pupa. This is a vital time of growth and development for the larvae.

Characteristics of third instar larvae are mentioned below:

Size and Appearance: Third instar larvae are bigger than first and second instar larvae. They are around 3-4 mm long and have a worm-like look. They have unique head, thoracic, and abdominal parts (Brandt et al., 2013).

Mouth Hooks: Third instar larvae have mouth hooks that they utilise to crawl and navigate around their surroundings (Grotewiel et al., 2005).

Moulting: Moulting occurs before the third instar larvae enter the pupal stage, during which they lose their outer cuticle to suit their developing bodies. They undergo the wandering stage after moulting, during which they travel away from the food supply in search of a suitable site to pupate (Matthews et al., 2021).

Pupation Preparation: Towards the end of the third instar, the larvae stop feeding and begin to look for a place to pupate. When they select a suitable location, they use their mouth hooks to anchor themselves and begin the pupation process (Thompson et al., 2021).

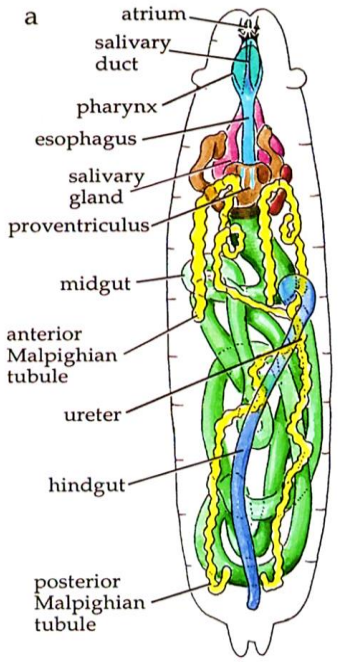


Figure 2.22: Schematic representation of anatomy and development of third instar larvae of *Drosophila melanogaster*, showing glands, ducts, digestive system, etc.

The transition from third instar larva to pupa is an important stage in *Drosophila melanogaster's* life cycle (Boomgarden et al., 2019).

2.13.3 Drosophila melanogaster as the model for neurodegeneration

Drosophila shares a significant proportion of genes associated with neurodegeneration, making it a valuable model for studying these diseases (Lenz et al., 2013). Fruit flies can express disease-related proteins implicated in neurodegenerative disorders, including tau and β-amyloid in AD, α-Syn in PD, and mutant huntingtin in HD (Chan et al., 2000). By expressing these proteins in specific tissues or neurons of fruit flies, researchers can study their effects on neuronal function and identify potential therapeutic targets. These models enable researchers to investigate the underlying mechanisms of disease progression and test potential interventions (Bolus et al., 2020).

Fruit flies have a well-annotated genome and their genetic composition is widely understood, despite being far simpler than humans (Wit et al., 2013). Many of their genes are comparable to those present in humans, especially those linked to neurodegenerative disorders. This enables researchers to modify and examine particular genes implicated in various disorders, providing vital insights into disease processes. *D. melanogaster* have a very short life cycle, allowing researchers to analyse numerous generations in a short amount of time (Zwaan et al., 1995). This is especially useful in neurodegenerative disease studies, where studying disease development and inheritance through generations is critical. Fruit flies offer a wide array of genetic tools and techniques that facilitate neurodegeneration research. For instance, researchers can use genetic manipulations, such as gene knockdown or overexpression, to modify specific genes of interest and study their effects on disease-related phenotypes. Additionally, techniques like RNA interference (RNAi) and CRISPR/Cas9 gene editing enable precise genetic modifications in *Drosophila*. Fruit flies exhibit a repertoire of behaviors that can be assessed to study neurodegenerative diseases (Hirth, 2010). Researchers can examine locomotor activity, learning and memory, olfactory responses, and circadian rhythms in fruit flies expressing disease-associated proteins or with genetic modifications (Luckinbill et al.,1984). These behavioral assays provide insights into the functional consequences of neurodegeneration and help identify potential therapeutic interventions. The relatively short lifespan and large number of offspring produced by fruit flies make them amenable to high-throughput screening approaches (Sang and Jackson, 2005). This allows researchers to test a large number of compounds or genetic interventions rapidly, screening for potential therapeutic agents or modifiers of disease progression. By leveraging the major improvements of

Drosophila as a model organism, researchers have made significant contributions to our understanding of neurodegenerative diseases (Laturney and Billeter, 2014). The use of *Drosophila melanogaster* as a neurodegenerative model has greatly aided our knowledge of the molecular processes underlying these disorders (Sun et al., 2002). Fruit flies have helped uncover disease mechanisms, identify potential therapeutic targets, and screen for candidate drugs, ultimately aiding in the development of treatments for these debilitating conditions (Bilen and Bonini, 2005). Researchers may investigate the implications of these genetic alterations on fly behaviour, brain function, and general health by introducing human genes containing AD-associated mutations into fruit flies. Amyloid beta plaques and tau tangles are aberrant proteins that accumulate in the brain during AD. In *Drosophila*, these pathological traits may be mimicked by expressing human amyloid beta or tau proteins with disease-causing mutations. This allows researchers to look at how these protein aggregates arise and how they affect neuronal function (Huber et al., 2020).

Altogether, the advancement of medicine and science has enabled a significant increase in global life expectancy. The growing proportion of the elderly will also result in a rise in the prevalence of age-related disorders, particularly neurodegenerative disorders. The nervous system is plagued by over 600 disorders, and the burden of these neurodegenerative disorders is escalating inexorably. There is currently no complete therapy or cure for neurodegenerative disorders. However, commercially available medications for neurodegenerative illnesses merely alleviate symptoms and do not prevent the underlying neurodegeneration that is linked with long-term negative effects. As a result, effective medicines capable of halting the gradual loss of certain neuronal populations are needed. On the other hand, plant-based nanoparticles are generally considered safe and well-tolerated. They are biocompatible and can be engineered to specifically target affected areas in the brain, minimizing off-target effects and reducing the risk of adverse reactions. They can carry and deliver therapeutic molecules, such as small interfering RNA (siRNA) or neurotrophic factors, directly to the brain. Additionally, these nanoparticles could be engineered to cross the BBB, allowing efficient drug delivery to the CNS. While plant-based nanoparticles hold promise for neurodegenerative diseases, It should be noted that further study is required to fully understand their therapeutic potential, optimise their qualities, and assure their safety and efficacy in human studies. Hence, the present investigation has been designed to access the neuroprotective efficacy of phyto-synthesized ZnO nanoparticles against rotenone-induced neurodegeneration.

CHAPTER-3

HYPOTHESIS

The term neurodegenerative diseases refers to a set of conditions that feature the gradual degradation of neurons in the nervous system and brain. These diseases have a significant impact on individuals' cognitive and motor functions, and their prevalence and incidence vary across different conditions and populations. The homeostasis among both antioxidants and oxidants regulates the redox status of cells. Any imbalance between these two that can result in necrosis or apoptosis determines the oxidative status of the cell. The key factor contributing to the increased oxidative stress sensitivity of brain cells is reactive oxygen species. Although oxygen (O_2) is typically a non-reactive molecule, it can be metabolized to form oxidation products including hydroxyl radicals (OH^-), superoxide anions (O^{2-}), and several other reactive species in the body. It leads to dreaded conditions including neurological diseases. Cellular defence systems that include endogenous antioxidants including glutathione reductase, catalase, glutathione peroxidase, and superoxide dismutase contribute in detoxification. Significant cell biomolecules including lipid, protein, and DNA are damaged by the degradation of these defence mechanisms, which may lead to alteration in cell signalling pathways. Systemic administration of drugs to the CNS is a substantial obstacle, owing to their short half-life, considerable first-pass digestion, restricted accessibility to the brain, and potential adverse impact when accessing non-target peripheral organs. As a result, developing systemic delivery mechanisms with higher potency is critical for CNS pharmacotherapy. Cholinesterase (ChE) inhibitors, tacrine, N-methyl-D-aspartate (NMDA) agonists, levodopa, or other dopaminergic agonists have never been utilized in conventional medicine or therapeutics to alleviate anything apart from motor symptoms by replenishing neurotransmitters. However, prolonged use of these medications can have substantial side effects, including other motor challenges. There is excessive activation of receptors for the neurotransmitter glutamate, resulting in an influx of calcium ions into neurons. Some conventional medications may inadvertently disrupt the delicate balance of glutamate and contribute to excitotoxicity, leading to neuronal damage or death. To stop or reduce the progression of numerous neurodegenerative disorders, researchers must create new natural neuroprotective agents due to the inadequacy of treatment medicines. By activating the Nrf2/Akt/PI3K cascade and eliminating free radicals, nutraceuticals and other phytonutrients have been proven to have protective benefits and to reduce the consequences of neurodegenerative conditions.

The World Health Organization (WHO) estimates that 78% of people worldwide currently use phytomedicine as their primary source of treatment. The Ayurvedic medicinal system has long employed the plants UD (*Urtica dioica*), MC (*Matricaria chamomilla*), and MK (*Murraya koenigii*) as well-known nerve relaxants and cognition boosters. These three herbs include phytochemicals with proven protective properties, including quercetin, alkaloids, vitamins, and polyphenols. Delivering drugs and dietary antioxidants to specific targets in the brain poses several obstacles, primarily due to the existence of the BBB, and other physiological factors in treating oxidative stress-related disorders, particularly neurodegen-erative diseases. Novel approaches for the improved crossing of the BBB must be developed in order to progress in the effective therapies. The use of novel nanotechnology-based methods, such as nanoparticles as nanocarriers may cross BBB and deliver the proper dosage of drug/medicine to the target brain region. Nanotechnology is a novel and fast evolving technique with several potential applications. It entails the fabrication and use of substances having one or more dimensions ranging from 1 to 100 nm. Using plant extracts from various plant species, many metals, including zinc oxide (ZnO), gold (Au) and many others, have been used in the biosynthesis of NPs. Zinc oxide high pharmacological qualities have enhanced its usage in healthcare applications such as antioxidant, antibacterial , and anticancer. Furthermore, zinc oxide, along with four other compounds of zinc, has been designated as generally recognised as safe (GRAS) compound by the US-FDA Food and Drug Administration. Even though UD, MC, and MK NPs have been utilized in various *in vivo* and *in vitro* research, there is currently less information available regarding the beneficial impact of these NPs on cellular and neurological issues.

This work used the green method to fabricate ZnO-NPs from the UD, MC, and MK fractions. The phyto fabricated ZnO-NPs were analysed utilizing SEM, FT-IR, UV-vis, Zeta potential, X-ray technique, EDX, and DLS method. The well diffusion approach was employed to investigate the anti-microbial activities of biosynthesized ZnO-NPs against *Staphylococcus aureus* and *Escherichia coli*. Moreover, we aimed to explore the therapeutic potential of phyto-fabricated ZnO-NPs against a commonly used neurotoxic organic pesticide rotenone *in vivo* in *Drosophila* (Oregon R^{+}) at neuronal, organismal, cellular, and molecular levels for the first time. We also evaluated its behavioural parameters on *Drosophila*, which has previously been proved to be a valuable indicator for neurodegenerative pathologies.

CHAPTER-4

AIM AND OBJECTIVES

AIM

The overarching goal of this study is to investigate the potential neuroprotective effects of phyto-synthesized zinc oxide nanoparticles (ZnO-NPs) in a nontarget *D. melanogaster* model that has been exposed to rotenone, a neurotoxin. Specifically, the research aims to target and modulate the levels or activity of proteins associated with neurodegenerative diseases, including *Amyloid-β*, *Tau*, and *α-Synuclein.*

OBJECTIVES

1. Determination of active components in phyto-extract viz. *Urtica dioica*, *Murraya koinegii,* and *Matricaria chamomilla* and their characterization using analytical methods.
2. Fabrication and characterization of green engineered zinc oxide (ZnO) nanoparticles using phytoextract of *Urtica dioica, Murraya koinegii,* and *Matricaria chamomilla*.
3. Generation of neurodegenerative model *Drosophila melanogaster* through chemical induction via rotenone treatment and biological validation using biochemical, cellular and molecular approach as well as classical organismal assays.
4. Assessment of modulatory effect(s) of green engineered ZnO nanoparticles on neurodegenerative model of *Drosophila*.

CHAPTER-5

MATERIALS AND METHODS

5.1 CHEMICALS AND REAGENTS

Acetylthiocholine iodide ($C_7H_{16}INOS$), Zinc nitrate hexahydrate (*$Zn(NO_3)_2 \cdot 6H_2O$), butyrylthiocholine iodide (*$C_9H_{20}INOS$), and rotenone ($C_{23}H_{22}O_6$) were purchased from Sigma (Roeder-mark, Germany). Folin Ciocalteu's reagent (C_6H_6O), propionic acid ($C_3H_6O_2$), trypan blue dye ($C_{34}H_{28}N_6O_{14}S_4$), phosphate buffer saline ($Cl_2H_3K_2Na_3O_8P_2$) 1,-1-diphenyl-2-picrylhydrazyl DPPH ($C_{18}H_{12}N_5O_6$), gallic acid ($C_7H_6O_5$), rutin ($C_{27}H_{30}O_{16}$), ABTS ($C_{18}H_{18}N_4O_6S_4$), quercetin ($C_{15}H_{10}O_7$), trichloroacetic acid ($C_2HCl_3O_2$), ascorbic acid ($C_6H_8O_6$), aluminum chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), sulfuric acid (H_2SO_4), ferric chloride ($FeCl_3$), ethidium bromide ($C_{21}H_{20}BrN_3$), ethanol (C_2H_6O), bovine serum albumin (BSA) ($C_8H_{21}NOSi_2$), Ellman's reagent ($C_{14}H_8N_2O_8S_2$), nutrient agar (*$C_{14}H_{24}O_9$),* chloramphenicol ($C_{11}H_{12}Cl_2N_2O_5$), potassium chloride (KCl), sodium benzoate ($C_7H_5NaO_2$), Tris-HCl buffer ($C_4H_{13}Cl_2NO_3$) acetone (C_3H_6O), methanol (CH_3OH), potassium persulphate ($K_2S_2O_8$), and sodium carbonate (Na_2CO_3) were acquired from Hi-Media (Mumbai, India).

The solvent used in HPLC testing was HPLC grade, while all other organic solvents and chemicals were analytical grade. The Ultraviolet Visible Spectrometer (Shimadzu UV-1601, Tokyo, Japan) was used for the colorimetric tests. Before use, all glassware was cleaned with deionized water and dried in the oven. To measure the fluorescent product, a microplate fluorometer (Fluoroskan Ascent system by Thermo Fisher Scientific) was used. To amplify the cDNA, the thermocycler (Applied Biosystems from Thermo Fisher Scientific) was used. The PCR product was performed on a gel electrophoresis device, and the Gel documentation system Model G5-0024 (Syngene, India) was used to capture/document DNA bands.

5.2 PLANT MATERIALS USED IN THE STUDY

Fresh *Urtica dioica* (UD) leaves were obtained from local apple orchards and kitchen gardens of Sopore (J&K, India), before the plants began to generate seeds. The Mediaroma Agro Company Ltd. (Kaskanj, UP, India) provided the *Matricaria chamomilla* (MC) flowers, while *Murraya koenigii* (MK) young leaves were acquired from Ayushya Vatika of the Lovely Professional University campus (Punjab, India). A taxonomist from the department of botany at the University of Jammu (Jammu, India) verified the identification and certification of the therapeutic plants.

Table 5.1: Scientific name and accession number of identified plants used in this study.

S. No.	Scientific Name	Common Name	Family	Accession number
1.	***Urtica dioica***	Nettle	Urticaceae	HBJU-17038
2.	***Matricaria chamomilla***	Chamomile	Asteraceae	HBJU-17039
3.	***Murraya koenigii***	Curry leaves	Rutaceae	HBJU-17040

5.2.1 Drying, processing, and extraction of samples

The collected plant leaves and flowers were washed under tap water and dried at room temperature for a week. After drying, to obtain fine particles, all dry plant components were crushed to a fine powder with a mechanical grinder and then sieved through a 40-micron sieve. A 10% aqueous extract was prepared using five grams (±0.05) of powdered sample in 50 ml deionized water and steeped at 95- 100°C for 10-15 min. Then, the fractions were filtered by using Whatman filter paper No. 1, and a 10% ethanolic extract was prepared using Soxhlet. Twenty grams (±0.05) of powdered plant samples were inserted into a Whatman 25 mm × 100 mm celluloid thimble by adding 200 ml of ethanol as a solvent at boiling temperature (70°C). A dark green colored extract was obtained for UD and MK leaves, whereas the extract obtained from MC flowers was pale yellow. The extract was then evaporated to dryness using a vacuum rotatory evaporator at 70°C or less to remove the solvents. The crude extracts were weighed and kept in an airtight dark container at 4°C for future analysis (Markom et al., 2007).

5.2.2 Determination of plant yield

The percentage yield of UD, MC and MK samples was calculated using the following formula (Adams et al., 2019):

$$W_2\text{-}W_1/W_0\times100$$

where W_2 (signifies the weight of the extract including the container), W_1 (signifies the weight of the container itself), and W_0 (signifies the weight of the initial dried sample).

5.2.3 ANTIOXIDANT ASSAYS

(a) Free radical-scavenging activity using DPPH assay

Antioxidant potential was evaluated using the 1,-1-diphenyl-2-picrylhydrazyl (DPPH) test (Baliyan et al., 2022). A freshly prepared solution of DPPH (0.011 gm) was taken in 50 ml

methanol for spectrophotometric measurements. The DPPH solution was further diluted with methanol. The optical density (OD) was adjusted between 0.8 and 1, and different plant fraction concentrations were applied to every 2 ml of DPPH mixture. Absorbance was measured at 517 nm using a Shimadzu UV-1601 spectrophotometer (Tokyo, Japan) after 30 minutes of incubation. Methanol was used as a blank, and DPPH was used as a control. Triplicate experiments were performed. Using the following equation, the radical scavenging activity was calculated as percent inhibition (1%) of the DPPH radical:

$$\text{DPPH inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where A_{sample} is the absorbance of the plant extract sample and $A_{control}$ is the absorbance of the DPPH solution as a control.

(b) ABTS radical cation decolorization assay

The 2, 2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation decolorization experiment was also used to examine the ability of plant extracts to scavenge free radicals, which is based on antioxidants in plant extracts reducing ABTS radicals (Wołosiak et al., 2021). The radical cation formed when the ABTS stock solution (0.036 g in 10 ml methanol) was mixed with potassium persulfate (0.057 g in 10 ml methanol) in a 1:1 ratio. Then, the mixture was kept in the dark for 16 hours at room temperature. The ABTS solution was further diluted with methanol to achieve an optical density (OD) of 0.8-1. Extracts of varying strengths were added to each 2 ml of ABTS solution. All samples were measured at 745 nm after 30 minutes of incubation. The percentage of scavenging activity was calculated using the following equation:

$$\text{ABTS inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

where A_{sample} signifies the absorbance of the plant extract sample and $A_{control}$ signifies the absorbance of the ABTS solution as a control.

(c) EC_{50} (Dose-response relationship)

An EC_{50} is a numerical analysis of the percentage of an antibody, drug, or poisonous chemicals that, after a certain amount of time, causes a reaction that is half as strong as the peak response. Data analysis for dose-response experiments and free radical scavenging qualities were performed by utilizing CompuSyn software to measure the potential of biosynthesized ZnO-

NPs from UD, MC, and MK, as well as their extracts. Higher radical scavenging potential is predicted by lower EC_{50} values (Chen et al., 2013).

5.2.4 DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC)

The total phenolic content of the plant extracts was measured by using Folin-Ciocalteu's colorimetric method (Clarke et al., 2013). Each plant extract was mixed with 2.5 ml of Folin-Ciocalteu's reagent (1:10 v/v) and vortexed. After 5 min, 2.0 ml of sodium carbonate (7.5%) was added. Then, the solution was incubated for approximately 90 min at room temperature before taking the OD at 760 nm by using a UV/Vis spectrophotometer. The results are given in milligrams of gallic acid equivalents per gram of dry weight (mg GAE/g). Triplicates of each sample were analyzed.

$$C = \frac{\mathrm{c} \times \mathrm{V}}{\mathrm{m}}$$

where 'C' indicates the total phenolic component content in (mg g^{-1}) plant extract in GAE, 'c' indicates the gallic acid concentration (mg ml^{-1}), 'V' indicates the volume of extract in microliters (µl) and 'm' indicates the weight of crude plant extract in grams.

The correlation coefficient (R^2) value was determined using the mean of three absorbance determinations for each concentration. The equation is shown below:

$$\mathrm{Y} = \mathrm{mx} + \mathrm{c}$$

where 'Y' signifies the extract's absorbance, 'm' signifies the slope from the calibration curve, 'x' signifies the extract's concentration, and 'c' is the intercept.

Concentrations of extracts were calculated using this regression equation. The phenolic content was estimated using the calculated value of each extract concentration.

5.2.5 DETERMINATION OF TOTAL FLAVONOID CONTENT (TFC)

Total flavonoids were quantified using the aluminum chloride colorimetric technique (Do et al., 2014). The plant extracts were mixed with 1.5 ml of methanol, 0.1 ml of (10%) aluminum chloride followed by 0.1 ml of potassium acetate (1 M), and finally 2.8 ml of deionized water. The reaction mixture was incubated for 40 min at room temperature, and the absorbance of the solution was obtained at 415 nm. Quercetin was used to create a calibration curve. The total flavonoid content was calculated in terms of quercetin equivalents (mg QE/g dry weight). Triplicate readings were taken for each plant sample.

$$C = \frac{\mathrm{c} \times \mathrm{V}}{\mathrm{m}}$$

where 'C' is the total phenolic content in mg g^{-1} plant extract in E, 'c' is the concentration of quercetin calculated from the calibration curve (mg/ml), 'V' is the volume of plant extract in µl and 'm' is the weight of crude plant extract in grams.

The absorbance of each concentration of the extract was measured using the procedure described above. Then, using the calculations above, the total flavonoid content was determined.

5.2.6 PRELIMINARY QUALITATIVE SCREENING OF PLANT EXTRACTS

Bioactive compounds such as polyphenols, reducing sugars, alkaloids, terpenoids, glycosides, flavonoids, saponins, and amino acids are mostly responsible for curative capabilities such as menstruation problems, muscular spasms, anemia, ulcers, hemorrhoids, inflammation, and wound healing (Sreenivasulu and Fernie, 2022). The presence of phytochemicals is determined using conventional qualitative test methods, which are as follows:

a) ***Detection of phenols (Ferric chloride test):*** In a test tube 2 ml of plant extract was treated with 2 ml of 5% ferric chloride aqueous solution. The presence of phenols was indicated by a deep blue–green solution (Sofowora, 1996).

b) ***Detection of flavonoids (Alkaline reagent test):*** A few drops of NaOH (20%) solution were added to 2 ml of plant extract, which displayed a yellowish red color within a second and turned transparent with the addition of dilute HCl, displaying a positive result (Chomnawang et al., 2005).

c) ***Detection of alkaloids (Wagner's test):*** To 4 ml of plant extract, 3 drops of Wagner's reagent were added. The appearance of a reddish-brown precipitate indicated a positive outcome (Chomnawang et al., 2005).

d) ***Detection of tannins ($FeCl_3$ solution test):*** By adding alcoholic ferric chloride (10%) solution into 2 ml of plant extract. The appearance of blue/green color suggested a positive outcome (Chomnawang et al., 2007).

e) ***Detection of carbohydrates (Molisch's test):*** Three milliliters of extract and 3 ml of H_2SO_4 were placed in a test tube; a few drops of Molisch's reagent were added (conc.). Allowing 3 min to stand and the appearance of a red/dull violet tone at the interphase of the two layers showed a successful outcome (Sofowora, 1993).

f) ***Detection of saponins (Saponins foam test):*** Five milliliters of distilled water were mixed with 0.5 ml of plant extract. The presence of saponins is indicated by foaming (formation of creamy tiny bubbles) (Olugbenga et al., 2022).

g) ***Detection of terpenoids (Salkowski test):*** Two milliliters of extract and a few drops of conc. H_2SO_4 was mixed with 1 ml of chloroform. The appearance of a reddish-brown precipitate indicated a positive outcome (Sofowora, 1993).

h) ***Detection of steroids (Liebermann-Burchard test):*** Three milliliters of acetic anhydride were mixed with 5 ml of plant extract. Then, 2 ml of H_2SO_4 was added to it. The presence of steroids is indicated by a shift in color from violet to bluish green (Olugbenga et al., 2022).

i) ***Detection of glycosides (Kellar-Kiliani test):*** One milliliter of glacial acetic acid was mixed with 2 ml of plant extract. Then, 1 ml of $FeCl_3$ and 1 ml of (conc.) H_2SO_4 was mixed into it. The appearance of glycosides was confirmed with the solution's greenish-blue color (Olugbenga et al., 2022).

5.2.7 HPLC CHROMATOGRAPHIC ANALYSIS OF PLANTS

A Shimadzu Prominence I LC2030 Plus HPLC system (Kyoto, Japan) equipped with a Shimadzu LC 2030 UV–VIS detector was used to separate natural compounds from crude extracts of UD, MC, and MK. The external standard technique was used to perform HPLC analysis under isocratic conditions. Before running in the column, the mobile phase was degassed and filtered through a membrane using methanol and 0.5% acetic acid in water (90:10 v/v). ColumnC-18 (4.6 mm ×250 mm) with a 5 µm particle size was used and maintained at 25°C temperature. Each injection volume was prepared at 20 µl and then injected into the HPLC. Samples were filtered using a 0.45 mm membrane filter (Millipore) before being put in vials, and a 1.0 ml/min flow rate was employed.

Spectral information was analyzed in the 200-400 nm region, and chromatograms were detected at a wavelength of 280 nm. Using previous documents, the quantitative quantification of each bioactive component contained in the plant extracts was determined (Pinelli et al., 2008; Miguel et al., 2015; Pandit et al., 2011). Peak identification was carried out by comparing the retention times of specific standards to the extract. The retention times of specific standards were compared to the extract to identify the peak.

5.3 BIOSYNTHESIS OF ZnO NANOPARTICLES

In order to synthesize ZnO-NPs for the experiment, modified green synthesis technique was used from previous study (Elumalai et al., 2015). Aqueous plant extract (UD, MC, and MK) of 25 ml was mixed with 0.3 mM of zinc nitrate hexahydrate ($Zn(NO_3)_2.6H_2O$) solution by constant stirring. Then 2M NaOH was added to the mixture to keep pH 12. The mixture was kept for stirring at 65˚C for two hours on a magnetic stirrer. After stirring, the mixture was collected and heated overnight in an oven at the same temperature until a thick yellow paste was obtained. This paste was then thoroughly dried and calcined at 400˚C for 2 hours. Subsequently, the pale white powder of ZnO NPs was obtained and used for further characterization. Calcination eliminates impurities from the sample, yielding a pure form of the NP; the process is temperature-dependent.

This procedure was performed again at temperatures of 55˚C and 75˚C, which were higher and lower than the operating temperature, respectively. At 55˚C, the reaction between Zn(NO3)2.6H2O and plant extracts did not occur, resulting in the creation of nano ZnO. In addition, because of the elevated temperature, when 75˚C was used as the operating temperature, it created ashes. At 65˚C , however, a yellow hue shift was seen with no deleterious consequences. Throughout the characterisation procedure, the sample was shown to be ZnO nanoparticles. As a consequence, temperature has a substantial influence on nanoparticle production. The light-yellow powder that resulted was used for structural, antioxidant, antibacterial, and in vivo research.

5.3.1 Optimization of various physicochemical parameters for biosynthesis of ZnO-NPs

Optimizing various physicochemical parameters for the biosynthesis of ZnO-NPs is important to control the size, shape, and properties of the nanoparticles. Here are some key parameters that was optimized during the biosynthesis process:

(a) Effect of plant extract concentration:

The concentration of the plant extract utilised in the synthesis process might impact the pace of reduction and subsequent nanoparticle production. Higher quantities of plant extracts may result in quicker reduction and nucleation, perhaps resulting in smaller nanoparticles. However, an extremely high concentration may cause aggregation or other unwanted effects. A 10% and 20% plant extract (UD, MC, and MK) was used to synthesize ZnO-NPs (Yusof et al., 2022).

(b) Effect of metal ion concentration:

The concentration of the zinc precursor, such as zinc nitrate influences the nucleation and growth of ZnO-NPs. Higher concentrations of the precursor may lead to the formation of larger nanoparticles. The metal ion concentrations were maintained at 0.1mM, 0.3mM, and 0.5mM. The quantity of leaves extract, temperature, pH, and incubation duration of the solution were all kept constant. The absorbance of the solution was measured at 300-500 nm (Asif et al., 2021).

(c) Effect of Temperature:

The kinetics of nanoparticle production are influenced by the reaction temperature and time. Higher temperatures might speed up the reduction process, resulting in faster nanoparticle development. Excessive temperature, on the other hand, might cause unwanted agglomeration or instability. The temperature was maintained at 55˚C, 65 ˚C, and 75˚C. The absorbance of the solution was determined using the spectrophotometric technique. The metal ion concentrations, reaction duration, and pH of the reaction mixture were all kept constant (Yusof et al., 2022).

(d) Effect of pH:

The pH of the reaction mixture plays a crucial role in nanoparticle synthesis as it affects the stability of the plant extract and the following reduction process. Different pH levels can cause changes in nanoparticle size, shape, and crystallinity. Using 2M NaOH, the pH was kept at 8.0, 10.0, and 12.0. The reaction mixture concentration, temperature, and reaction time were all kept constant. The absorbance of the solution was determined using the spectrophotometric method. The method was performed three times to optimise the pH (Asif et al., 2021).

(e) Effect of Reaction time:

The stability of the solution was measured at room temperature at intervals of 0.5, 1, 2, and 4 hours to investigate the influence of reaction time on the synthesis of ZnO-NPs (Yusof et al., 2022).

5.3.2 *Microscopy techniques for characterization of phyto- synthesized ZnO nanoparticles*

Microscopy techniques are an important tool for characterization which provides precise information about the shape, size, structure, composition, and spatial resolution of ZnO-NPs. The following techniques were used for the structural, morphological, and molecular identification of ZnO-NPs.

(a) UV-visible spectroscopy

The optical properties of synthesised ZnO-NPs of UD, MC, and MK and their extracts were observed using absorption spectra of NPs. This is recorded with an Ultraviolet Visible Spectrometer (Shimadzu UV-1601, Tokyo, Japan) with a wavelength range of 200-800 nm. The surface plasmon resonance (SPR) was generated by the interaction of light and the mobile surface electrons of ZnO-NPs. Previous research has established the peaks at 289-385 nm as the SPR of ZnO-NPs. The SPR band of ZnO-NPs determines their morphological features. The volume of plant extracts and other reaction conditions could also produce a shift in the SPR bands of ZnO-NPs. UV-Vis spectra were measured in a quartz cuvette with a path length of 1 cm at room temperature (Rajakumar et al., 2018).

(b) Powder X-Ray Diffraction

The X-ray diffraction (XRD) technique is recommended for determining the crystalline size, stress, and crystalline phase identification of Plant extracts and their synthesized ZnO-NPs (Abdelbaky et al., 2022). Analyses of XRD were carried out with a Bruker D8 Advance diffractometer using a Cu $K_{\alpha 1}$ radiation (λ = 1.54060Å, 30 mA, 45 kV). The diffractograms were obtained at 2q in the 20˚-90˚ range, with step-size of 0.05˚ and scan period of 2 s per step. During XRD evaluation, synthesized ZnO-NPs of UD, MC, and MK were subjected to intense rays from the XRD machine, which penetrate through them and provided useful information about their structure. The nano size is indicated by the broadening of the XRD pattern. The average size of ZnO-NPs is estimated using Debye-Scherer's equation.

$$d = k\,\lambda/\beta\cos\theta$$

where, d denotes the size of ZnO-NPs in nanometres, k denotes the Scherrer constant (0.9), λ observed x-ray wavelength, β indicates whole width at half maximum of the diffraction peak, θ observed Bragg angle. Sharp peaks at lattice planes (100), (002), (101), (102), (110), (103), (112), and (202) have been reported to reflect the crystallinity and purity of ZnO-NPs.

(c) Fourier transform infrared spectroscopy

Fourier transform infrared spectrophotometry (FT-IR) is an effective technique for detecting functional groups, chemical bonds, and molecular structures in substances (Ismail et al., 2013). As depicted in the annotated spectrum, the absorbed wavelength of light indicates the chemical bond. The chemical bonds of a molecule can be determined by analysing the infrared absorption spectra.

To record the functional groups involved in the ZnO-NPs produced from UD, MC, and MK their extracts, FTIR- spectrophotometer Perkin-Elmer Spectrum 2 (ATR and Pellet accessories) was used. A 10 mg of ZnO-NPs sample was contained in 100 mg of KBr pellet to generate translucent sample discs. The ZnO-NPs from each plant material were then submitted to FT-IR spectrophotometer. At room temperature, the samples were analysed in the infrared band with a spectral range of 400 cm^{-1} to 4000 cm^{-1} with a resolution of 4 cm^{-1}.

(d) Zeta potentiometer

The determination of zeta potential is a significant technique of nanocrystals for estimating surface charge in colloidal solution. Stability of synthesized ZnO-NPs of UD, MC, and MK was analyzed by using Particle size and Zeta potential analyzer (Malverrn Zeta sizer Nano ZS90). Due to electrostatic repulsion of individual particles, nanocrystals with a large positive or negative zeta potential imply good physical stability of NPs. A zeta potential value other than -30 mV to +30 mV is generally believed to have sufficient repulsive power to achieve superior physical colloidal stability (Clogston and Patri, 2011).

(e) Dynamic light scattering analysis

The particle size was calculated using the dynamic light scattering (DLS) technique with the Nano plus (Micromeritics, USA). The instrument can measure the particle size of samples suspended in liquids in the range of 0.1 nm to 12.2 m with sample suspension concentrations ranging from 0.00001 to 40%, and it has a sensitivity for molecular weight as low as 250 Da. The particle size (multimodal size distribution) was calculated by measuring the angles at which an incident light beam was scattered as a function of the colloidal particles' Brownian motion (Lim et al., 2013).

(f) Scanning electron microscopy analysis

The scanning electron microscope (SEM) is one of the most extensively used technique for characterisation of NPs. The signals produced by electron-sample interactions provide information about the sample, such as its surface morphology and chemical composition. The morphology of the synthesized ZnO-NPs of UD, MC, and MK was studied using a JEOL JSM-7610F Plus with an Au Sputter Coater. The samples were coated with a thin layer of gold or gold-palladium alloy to prevent charging of the surface, to enhance the emission of secondary electrons so that the specimen conducts evenly, and to offer a homogeneous surface for investigation and imaging (Mandal et al., 2022).

(g) Energy-dispersive x-ray spectroscopy

The presence of elemental zinc and oxygen was detected using an OXFORD EDS LN2. The samples were dried at room temperature before being tested for the composition of the generated ZnO-NPs. The EDX spectrum reveals the purity and composition of the UD, MC, and MK green synthesised ZnO-NPs. The EDX spectrum shows strong signals from the Zn element and light signals from the O, K, and Cu elements (Barzinjy et al., 2020).

5.4 MICROORGANISMS AND FLY STRAIN

A wild-type *Drosophila melanogaster* strain (Oregon R^+) that was kindly gifted by Dr. Anurag Sharma, Senior Assistant Professor, NITTE (Deemed to be University), Mangalore, India, and maintained on a standard diet containing yeast, agar, maize flour, propionic acid, sodium benzoate, and sulfur-free sugar. The flies were cultured at 24±1˚C, 12-h light/dark cycle, and 65-70% relative humidity (Singh et al., 2009). *Escherichia coli* and *Staphylococcus aureus* bacterial strains were cultured in sterile conditions at Lovely Professional University's laboratory (Phagwara, India).

5.4.1 *Antimicrobial assay*

The antibacterial activity of green engineered ZnO-NPs was evaluated against different bacteria, including *Escherichia coli* and *Staphylococcus aureus*. The bacteria were cultured on nutritional agar medium for 24 h at 37 °C. One colony of bacteria were selected and suspended in 5 mL of physiological Serum using a sterile inoculating loop. The turbidity of the bacterial isolates was adjusted to match McFarland criteria of 0.5. Inoculum tubes were swabbed with sterile swabs. By streaking the swabs across the Mueller Hinton agar plates, bacteria were inoculated. The well diffusion method was used for the antibacterial test. Each Petri plate had three wells (3 mm in diameter) that were filled with negative control (deionized water), positive control (chloramphenicol), and 100 µg of ZnO-NPs. The zone of inhibition on the plates was measured as a microbial species inhibition (Ifeanyichukwu et al., 2020).

5.4.2 *Optimized concentration of biosynthesized ZnO-NPs and rotenone exposure*

Preliminary tests were conducted with small numbers of *Drosophila* flies to see whether the treatment had any effect on the survival of the flies over the experimental period, testing several doses (0.01, 0.025, 0.05, and 0.1%) of biosynthesized ZnO-NPs of UD, MC, and MK. However, from the conclusive studies, only one concentration, 0.1% per unit of medium, was selected as the best concentration. Furthermore, the concentration of rotenone (500 µM) used

to assess the cellular and neuroprotective properties of UD-ZnO, MC-ZnO, and MK-ZnO NPs was selected based on our findings in *Drosophila* and those of other published reports (Akinade et al., 2022; Hosamani and Muralidhara, 2009; Kumar et al., 2022).

5.4.3 *Treatment and experimental groups*

D. melanogaster flies/third instar larvae were split into ten groups for the experiment. There were two control groups: Group I was fed standard *Drosophila* larval diet as a control, and Group II was administered food mixed with 0.1% DMSO as a vehicle control. Group III had ROT (500 M) alone; Group IV was ROT with UD-ZnO NPs (0.1%); Group V was ROT with MC-ZnO NPs (0.1%); and Group VI was ROT cotreated with MK-ZnO NPs (0.1%).

Table 5.2: Schematic representation of treatment regime in *in-vivo* studies

Groups	***Treatments***
Group I	Control (Untreated)
Group II	DMSO as a vehicle control (0.1%)
Group III	Zinc nitrate alone (0.1%)
Group IV	ROT (500 µM) alone.
Group V	ROT(500 µM) + UD Extract (0.1%)
Group VI	ROT (500 µM) + UD-ZnO NPs (0.1%)
Group VII	ROT (500 µM) + MC Extract (0.1%)
Group VIII	ROT (500 µM) + MC-ZnO NPs (0.1%)
Group IX	ROT (500 µM) + MK Extract (0.1%)
Group X	ROT (500 µM) + MK-ZnO NPs (0.1%)

*The symbols stand for DMSO (Dimethyl sulfoxide), ROT (Rotenone); UD (*Urtica dioica* extract); UD-ZnO (*Urtica dioica* synthesized zinc oxide nanoparticles); MC (*Matricaria chamomilla* extract); MC-ZnO (*Matricaria chamomilla* synthesized zinc oxide nanoparticles), MK (*Murraya koenigii* extract*);* MK-ZnO (*Murraya koenigii* synthesized zinc oxide nanoparticles).

Larvae were allowed to feed normally or with food that had been exposed to ROT or ROT+ZnO NPs of UD, MC, and MK for 24 and 48 hours, respectively. The flies were exposed for 120 hours (5 days) and their climbing and jumping abilities were evaluated. We examined the

modulatory properties of biosynthesized ZnO nanoparticles on rotenone-induced mortality, acetylcholinesterase inhibition, locomotor dysfunctions, cellular and proteotoxicity.

5.4.4 CELLULAR ASSAY

5.4.1 Trypan Blue Dye Exclusion Assay

Dye exclusion assay was used to determine cell viability with minor modifications as described by Krebs and Feder (1997). This is a quick and simple method for differentiating between living and non-living cells in tissue. It is used to evaluate cell death in the whole larval body and gut. It is based on the membrane impermeability of blue dye, live cells have an intact cell membrane, trypan blue cannot pass through the cell membrane and enter the cytoplasm. At the end of treatment, 8-10 larvae were washed three times with 0.1 M phosphate buffer saline (pH 7.4), then entire or dissected midguts of larvae were incubated in trypan blue solution (0.4%) for 15 minutes, followed by three washes with 0.1 M PBS. A stereomicroscope was used to examine the larvae; pictures were taken for trypan blue scoring and thoroughly scrutinised (Singh et al., 2010).

5.4.5 NEUROCHEMICAL MARKERS

(a) Preparation of D. melanogaster Brain Homogenates

With slight modifications, the *Drosophila* brain homogenates were prepared as previously reported**.** To prepare 10% homogenate, the head of third instar larvae of *Drosophila* from the control (untreated/ normal), DMSO, zinc nitrate, ROT, and ROT with plant extracts (UD, MC, and MK) and their biosynthesized ZnO-NPs groups were dissected and homogenised in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl. After homogenization, the samples were centrifuged for 15 min at 10,000×g at 4˚C. The supernatant was then filtered through a nylon mesh sieve with particle sizes of 10 mm before being used for neurochemical markers (Hosamani and Muralidhara, 2009).

(b) Acetylcholinesterase (AChE) Enzymatic Assay

AchE activity was determined in *Drosophila* fly brain tissues using the method reported previously with slight changes (Ellman et al., 1961). In brief, a 10% homogenate of brains from control and treated flies was produced in 50 mmol L^{-1} Hepes buffer with protease inhibitor, followed by a 15-min centrifugation at 10,000×g. The test combination included tissue homogenate, phosphate buffer, 5,5-dithiobis(2-nitrobenzoic) acid (DTNB), and

acetylthiocholine iodide as the substrate. The breakdown of acetylthiocholine iodide was measured at 412 nm and the findings were represented as mol min^{-1} mg^{-1} protein.

(c) Butyrylcholinesterase (BChE) Enzymatic Assay

BChE activity was determined in *Drosophila* fly brain tissues using the method reported previously with slight changes (Ellman et al., 1961). In brief, a 10% homogenate of brains from control and treated flies was produced in 50 mmol L^{-1} Hepes buffer with protease inhibitor, followed by a 15-min centrifugation at 10,000×g. The test combination included tissue homogenate, phosphate buffer, 5,5-dithiobis(2-nitrobenzoic) acid (DTNB), and butyrylthiocholine iodide as the substrate. The breakdown of butyrylthiocholine iodide was measured at 412 nm and the findings were represented as mol min^{-1} mg^{-1} protein.

5.4.6 BIOCHEMICAL ASSAYS

(a) Homogenate Preparation

To prepare 10% cytosol/homogenate, the tissue of third instar larvae of *Drosophila* from the control (untreated/ normal), DMSO, Zinc nitrate, ROT, and ROT with plant extracts (UD, MC, and MK) and biosynthesized ZnO-NPs groups were dissected and homogenised in ice-cold 0.1 M phosphate buffer (pH 7.4) containing 0.15 M KCl. After homogenization, the samples were centrifuged for 15 min at 10,000×g at 4˚C. The supernatant was then filtered through a nylon mesh sieve with particle sizes of 10 mm before being used for various biochemical tests (Shabir et al., 2022).

(b) Estimation of Catalase (CAT) activity

The ability of the enzyme to split H_2O_2 within 1 min of incubation in the reaction mixture was used to calculate CAT activity (Deepashree et al., 2022). In a tube, 100 µL of 10% tissue homogenate, 400 µL distilled water, and 1.0 mL of 0.01 M phosphate buffer (pH 7.0) were mixed together. The reaction was begun by adding 0.5 mL of 0.2 M H_2O_2 and incubating it at 37 C for 1 min. The reaction was halted by adding 2.0 mL of 3:1 acetic acid and 5% $K_2Cr_2O_7$. This resulted in the immediate formation of blue per chromic acid precipitate.

The reaction mixture was immediately placed over a boiling water bath (95-100ºC) for 15 min, resulting in the synthesis of chromic acetate with a stable green colour. The reaction mixture was allowed to cool before measuring the absorbance of the coloured result at 570 nm against

a blank that included distilled water as a homogenate replacement. The activity was expressed in terms of µmoles H_2O_2 min^{-1} mg larval $protein^{-1}$.

(c) Estimation of superoxide dismutase (SOD) activity

SOD activity was evaluated in third instar larvae of *Drosophila* (n=3, 20 larvae/group) using a previously developed technique with slight modifications (Musachio et al., 2022). In brief, 20 third instar larvae were homogenized in 0.5 mL of sodium phosphate (0.1 M) buffer containing 0.15 M KCl (pH 7.4) and centrifuged for 10 minutes at 4°C at 10,000 rpm. At room temperature, 0.2 mL of NADH (780 µM) was added to 1 mL of 0.052 M sodium pyrophosphate buffer (pH 8), 0.3 mL of nitrobluetetrazolium, 0.1 mL of 186 µM phenazine methosulphate, 200 µL of milliQ water, and 0.2 mL of supernatant from larval homogenate.

After 1 minute at room temperature, the reaction was halted by adding 1 mL of acetic acid, and the colour intensity of the chromogen was measured at 560 nm. Each sample was done in duplicate, and each dosage contained three food vials. The enzyme activity necessary to suppress chromogen formation by 50% at room temperature was represented by one unit of enzyme activity, and the final findings of three such tests were given in units/min.

The SOD activity was calculated using the following formula:

$$\text{SOD activity} = [(A_{blank} - As_{ample}) / A_{blank}] \text{ x (dilution factor / reaction volume)}$$

Where, A_{blank}: Absorbance of the blank tube and A_{sample}: Absorbance of the sample tube.

(d) Estimation of lipid peroxidation (LPO)

The method of Ohkawa et al. (1979) was used to test MDA content using tetraethoxypropane as an external standard. In a test tube, 25 µL of 10% tissue homogenate was mixed with 0.15 mL of 10% sodium dodecyl sulphate (SDS), 825 µL of distilled water and the mixture was incubated at 24±1ºC for 5 min before the addition of 1.0 mL of 20% acetic acid solution (pH 3.5). The reaction mixture was allowed to stand at 37˚C for 5 min before adding 1.0 mL of 0.8% thiobarbituric acid (TBA) and heating it over a boiling water bath (95-100ºC) for 1 hour.

The reaction mixture was cooled, the final amount of distilled water was increased to 4.0 mL, n-butanol (4.0 mL) was added, and the entire mixture was shaken vigorously. After centrifuging at 4000×g for 10 min at 24±1ºC, the organic layer was separated and transferred to a new tube. At 532 nm, absorbance was measured against n-butanol. The amount of lipid peroxide was quantified in terms of n moles MDA formed h^{-1} mg larval $protein^{-1}$.

(e) Estimation of glutathione (GSH) content

The GSH content was determined colorimetrically using Ellman's reagent (DTNB), as described by Jollow et al. (1974). The test was based on the formation of yellow colour when 5, 5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added to sulphydryl-containing compounds. The GSH content was determined colorimetrically using Ellman's reagent (DTNB) using the technique published by Jollow et al. (1974). In a 1:1 ratio, the supernatant was precipitated with 4% sulfosalicylic acid.

The samples were stored at 4ºC for an hour before being centrifuged at 4500×g for 10 min at 4ºC. The test mixture contained 550 µL of phosphate buffer (0.1 M), 100 µL of DTNB and 100 µL of supernatant. The OD was measured at 412 nm and the findings were represented as moles of GSH/gram tissue.

(f) Estimation of Protein Carbonyl (PC) content

The protein content in the tissue homogenate was approximately 1 mg/mL. About 2.5 mL of 10 mM 2, 4-dinitrophenyl hydrazine (dissolved in 2.5 M HCl) was added to it, vortexed, and stored in the dark for 20 min. After that 125 µL of 50% (w/v) trichloroacetic acid (TCA) was added, properly mixed, and incubated at -20°C for 15 min. The tubes were then centrifuged at 8000×g for 10 min at 4°C (Singh et al., 2010).

(g) Protein Estimation Assay

The total protein concentration in the midgut of *D. melanogaster* homogenate was measured using the Lowry et al. (1951) method with bovine serum albumin (BSA) as the reference protein. With Folin's reagent, the phenolic group of tyrosine and tryptophan residues in a protein generates a blue purple colour complex with greatest absorption in the 660 nm wavelength region. As a result, the intensity of colour relies on the number of aromatic amino acids present and consequently differs between proteins.

To 0.5 ml of cytosol/homogenate sample, 500 µl of 10% TCA was added and centrifuged for 10 min. The precipitate was dissolved in 1000 µl of 0.1 N sodium hydroxide. An aliquot of 500 µl was removed from this, then 4.5 ml of alkaline copper reagent was added and left at room temperature for 10 min. After 20 minutes, 0.5 ml of Folin's phenol reagent was added, and the absorbance was measured at 640 nm. The values are given in milligrams per decilitre (mg/dl).

5.4.7 ORGANISMAL/BEHAVIORAL ASSAYS

(a) Negative Geotaxis Assay (Climbing Assay)

Anesthetized test flies were placed in a vertical glass column measuring 25 cm in standard length and 1.5 cm in diameter (Singh et al., 2022). The flies were gently tapped to the bottom of the column after a brief recuperation period. Flies that reached the top of the column and those that stayed at the bottom were counted separately after 1 minute.

The data was reported as a percentage of flies that escaped beyond a minimum distance of 6 cm every 60 seconds. For the experiment, twenty adults were employed each replication. The experiments were repeated three times, and the score for each replication was the average of three trials for each insect group, including the control. For each experiment, a performance index (PI) was calculated and presented as follows:

$$1/2[(n_{tot} + n_{top} - n_{bot})/n_{tot}]$$

(b) Memory assay

This test was performed on the flies after raising them in the conditions that was utilized as a learning condition to perform memory assay (Ali et al., 2011). Take 10 flies in each vial in triplicate for the assay (total of 30 flies) in every group. Transfer the flies for half an hour in the food+dark and no-food + light conditions for eight hours. Flies were maintained in light after being provided the conditions. These constraints were imposed for three days. Flies were deprived for an hour on the fourth day before the analysis began. These constraints were imposed for a period of three days. Flies were deprived for an hour on the fourth day before beginning the analysis.

After one hour, food will be placed on one side of the T-maze that is dark, and no food on the other side that is bright, and the number of times the fly will avoid or follow the taught circumstances will be recorded. The experiment was not performed immediately since learning was not assessed, but rather for memory conditions influenced by neurodegenerative disorders. Examine the number of flies that arrive at the T and go to the designated sides. For the remaining one minute, repeat the procedure 10 times with the same vial. Record the number of flies as the percentage of the total number of flies advances towards light and dark. The experiment was done 5 times separately.

(c) Jumping Assay

The jumping activity was carried out to assess neuromuscular function. The rate of locomotor activity appears to influence the threshold for the jumping response. Newly emerged flies were transferred one at a time to a vial labelled 1-10 cm, and the distance jumped by the fly was measured from the bottom of the vial.

The mean number of jumps across five replicates was used to calculate the jumping activity. There were 100 flies in each group, with five replicates in each group (Sharma et al., 2012).

(d) Larval crawling Assay

The larval crawling behaviour is a basic experiment for understanding the rhythmic behaviour of the larva and detecting any neurological abnormalities (Sood et al., 2019). Third instar larvae of *Drosophila* was used to record the crawling behaviour. In order to clear the cuticular dietary substances, larvae were rinsed in phosphate-buffered saline (PBS).

The larvae were allowed to crawl on 2% agar plates prepared for the larval crawling experiment. A graph paper was placed beneath the plate to record the distance travelled by the larva. The larval trailing route was noted, and crawling speed was determined based on the time it took each larva to reach the agar plate's periphery. The speed was measured in millimetres per second on average.

5.4.8 Eye imaging of D. melanogaster under Stereo microscope

The control flies and the treated flies with ROT (500 µM) alone or in association with plant extracts (UD, MC, and MK) and their biosynthesized ZnO nanoparticles (UD-ZnO, MC-ZnO, and MK-ZnO) were examined for any changes in the appearance of the eyes. After treatment, adult flies were transferred and kept on a petri plate over an ice pack . Once the flies are anesthetized, transferred them to a clean surface (microscope slide).

This immobilizes the fly and allows for easy positioning under the microscope. Placed a drop of PBS on a microscope slide to create a wet mount. This will help keep the eyes hydrated and provide a clear view under the microscope. Captured images of the *Drosophila* eyes using an attached camera connected to the stereo microscope.

5.4.9 MOLECULAR PARAMETERS

(a) RNA extraction for Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) gene expression studies:

Brain ganglia (50 mg) of third instar larvae of Oregon R^+ were explanted in PSS and total RNA was extracted using 1 ml of RNA-XPress reagent (Hi-Media, India) and homogenized. The samples have been kept at room temperature for 5 minutes to allow the nucleoprotein complexes to completely dissociate. Following that, for each sample, 0.2 mL of chloroform was added. Incubated for 10 minutes at ambient temperature (15-20˚C), then centrifuged for 15 minutes at 13,000xg at 4˚C.

To precipitate RNA, the upper aqueous layer was moved to a new tube and 500 µL of isopropyl alcohol was added. Incubated at room temperature for 10 minutes after thoroughly mixing. Centrifuged at 13,000 x g for 10 minutes at 4˚C. RNA had been precipitated. The supernatant was discarded, and the RNA pellet was rinsed with 1 ml of 75% ethanol. Dry the RNA pellet by air-drying for 10 minutes. The pellet was resuspended in 50 µL of RNase-Free Water and kept at -80 C.

(c) Complementary DNA (cDNA) synthesis:

The fundamental procedure involves the catalytic conversion of an RNA template to a single stranded cDNA in the presence of primers (1 mg/ml) and dNTPs (5mM). A blend of oligo dT and random hexamers was used to prime the RNA template. Extracted RNA (2 g) was used in a total volume of 20 µL for cDNA synthesis, which included 4 µL of 5x reaction mix, 1 µL of reverse transcriptase, and the final volume was reached by adding RNase free water. The reverse transcription mixture was incubated for 5 minutes at 25˚C, 30 minutes at 42˚C , and 5 minutes at 85 ˚C.

(d) Polymerase chain reaction (PCR):

PCR amplification was performed in a thermocycler (Applied Bio systems from Thermo Fisher Scientific) utilising primer pairs specific for inducible forms of APP, Tau, and α-Syn. Primers for APP, Tau, α-Syn, and GAPDH were reported in their respective papers (Kong et al., 2020; Abul et al., 2018). Table 3 shows the primer pairs derived from Puregene (Madhya Pradesh, India).

Table 5.3: List of the primers used for PCR.

Target gene	Primer sequences (5'→3')	Amplicon size (bp)
APP	Forward: AACCGACTCCAGGATGACTATG Reverse: TCTGGGGTTCCATGTAAAAGC	215 bp
Tau	Forward: TGGGGAACATTCCGTATGAGG Reverse: CAGAAGCCATAACCCTTGGG	105 bp
α-Syn	Forward: TGTAGGCTCCAAAACCAAGG Reverse: TGTCAGGATCCACAGGCATA	164 bp
GAPDH	Forward: TGGATTTGGACGCATTGGTC Reverse: TTTGCACTGGTACGTGTTGAT	159 bp

*****APP (Amyloid precursor protein), Tau (Tubulin associated unit), α-Syn (*Alpha-synuclein),* GAPDH (Glyceraldehyde-3-phosphate dehydrogenase), *and bp (Base pair).*

PCR reaction mixture (total 25 µL) consisted of 1×Taq buffer, 1.5 mM $MgCl_2$, 0.20 mM dNTPs mixture, 0.40 µM each of forward and reverse primer, 1 U Taq DNA polymerase, milli-Q water, and 2 µL cDNA. Optimized PCR conditions consisted of an initial cycle of 94˚C for 3 min (denaturation), followed by 35 cycles (APP, TAU, α-Syn, and GAPDH) of 94˚C for 30 s (denaturation), 58˚C for 30 s (annealing), 72˚C for 45 s (extension) and a final step at 70˚C for 5 min (termination). After PCR, the amplicons were separated on a 1% agarose gel containing ethidium bromide at 5 V/cm and visualized with a GEL DOC Imaging System Model G5-0024 (Syngene, India). The size of the PCR product was determined by comparison to a DNA size marker. The intensity of the bands was quantified by the *ImageJ* Software of Bethesda, Maryland, USA. Each experiment was carried out with three biological replicates prepared from independent pools.

5.5 Data analysis

Each experiment was repeated three times, and the findings are reported as mean ± standard deviation (SD). CompuSyn software (version 1.0) was used to perform the EC_{50} analysis. The statistical analysis was carried out using SPSS software (version 18) and a one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference test. A p value of <0.05 or less was used to determine significance.

CHAPTER-6

RESULTS

The results of several assays and analyses used to determine the feasibility of UD, MC, and MK extracts against rotenone-induced lethality, locomotor dysfunctions, inhibition of acetylcholinesterase, cellular toxicity, proteotoxicity, and down expression of genes are presented in the subsections below.

6.1 Percentage yield of plant extracts

The results obtained show that the MK leaf extraction yield was higher than that of UD and MC when distilled water was used as the extracting solvent **(Table 6.1)**. However, the yield of ethanolic extraction was again higher in MK, followed by MC and UD. The findings also revealed that the variation in the yield depends on the extraction solvent used. The variability in extract quantities from the extracted plant materials in this study could be related to the varying accessibility of extractable components caused by plant chemical composition.

Table 6.1: Percentage yield of plant extracts using aqueous and ethanolic extraction methods.

S. No.	Plant species	Code	Plant part used	Solvents (%) yield (w/w)	
				Aqueous extracts	**Ethanolic extracts**
1.	***U. dioica***	UD	Leaves	14.34	10.45
2.	***M. chamomilla***	MC	Flower	8.56	14.68
3.	***M. koenigii***	MK	Leaves	21.62	15.27

6.2 Antioxidant potential of UD, MC, and MK extracts through 2,2-diphenyl-1-picrylhydrazyl and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) methods

The DPPH and ABTS assays are widely used methods to evaluate the antioxidant activity of plant extracts. These tests are based on the ability of antioxidants to scavenge free radicals and reduce their concentration, indicating their potential to protect against oxidative stress.

The DPPH assay measures the ability of an antioxidant to donate an electron or hydrogen atom to the stable free radical DPPH, which results in the reduction of DPPH and a change in its color from purple to yellow. The intensity of color change is proportional to the antioxidant activity. The antioxidant potential of both aqueous and ethanolic extracts of UD, MC, and MK

was measured using a free radical DPPH assay. As shown in **Figures 1(a,c)**, MK had the highest DPPH radical scavenging activity in both aqueous and ethanolic extracts, followed by UD and MC, which had the lowest scavenging activity. When compared to aqueous extracts, ethanolic extracts of all three plants revealed the strongest scavenging efficacy.

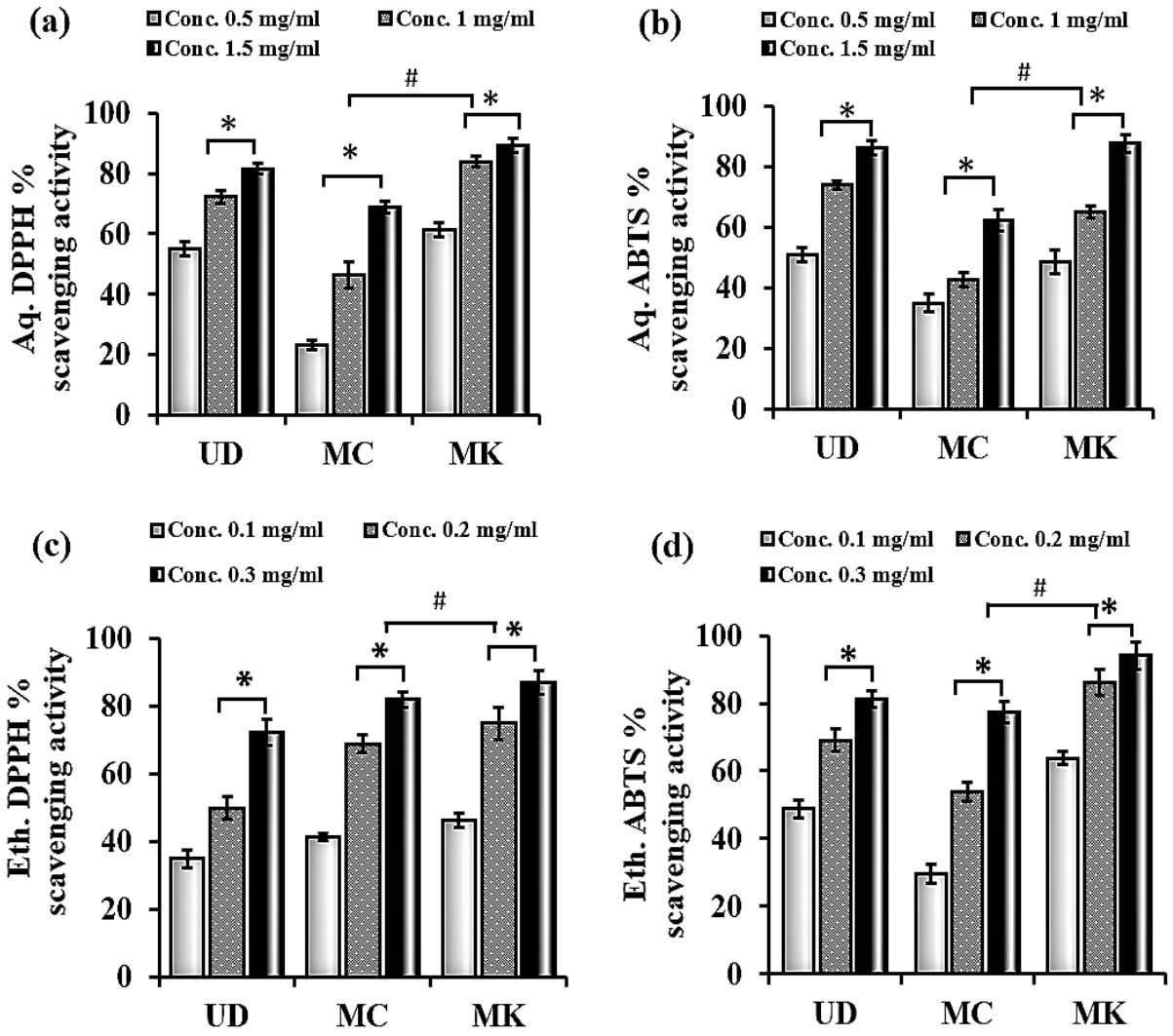


Figure 6.1: The effect of different concentrations of individual aqueous (a and b) and ethanolic extracts (c and d) of *U. dioica* (UD), *M. chamomilla* (MC), and *M. koinegii* (MK) in the DPPH (a and c) and ABTS (b and d) free radical scavenging test. Data are shown as the mean ± SD for n = 3. Statistically significance is ascribed as **P*<0.05 (intra group) and #*P*<0.05 (inter group) compared with 0.5 mg/ml and 0.1 mg/ml of the respective groups.

The ABTS assay measures the scavenging activity of antioxidants against the ABTS radical cation. In this test, the ABTS radical is generated by the reaction between ABTS and a specific oxidizing agent. The reduction of $ABTS^{+}$ by antioxidants results in a decrease in the absorbance, indicating their antioxidant capacity. The ABTS radical scavenging activity of different extracts with different extraction solvents (aqueous and ethanol, **Figure 1(b,d)**) was

found to be higher in MK, followed by UD and MC. This result suggests that MK has higher efficacy in scavenging free radicals along with the antiradical and antioxidant activity.

6.3 EC_{50} (half maximal effective concentration) prediction

The EC_{50}, also known as the half-maximal effective concentration, is a measure of the concentration of a compound or substance required to achieve a response that is halfway between the baseline (control) and the maximum response. The EC_{50} value is commonly used in pharmacology and toxicology to assess the potency or efficacy of a compound. In dose-response curves, the EC_{50} value represents the concentration at which the compound produces 50% of its maximal effect. It provides an indication of the compound's potency, as a lower EC_{50} value indicates a higher potency (i.e., a lower concentration is needed to achieve the desired effect). The EC_{50} can be calculated by interpolating data from a suitable curve or by performing a nonlinear regression of the data using different components.

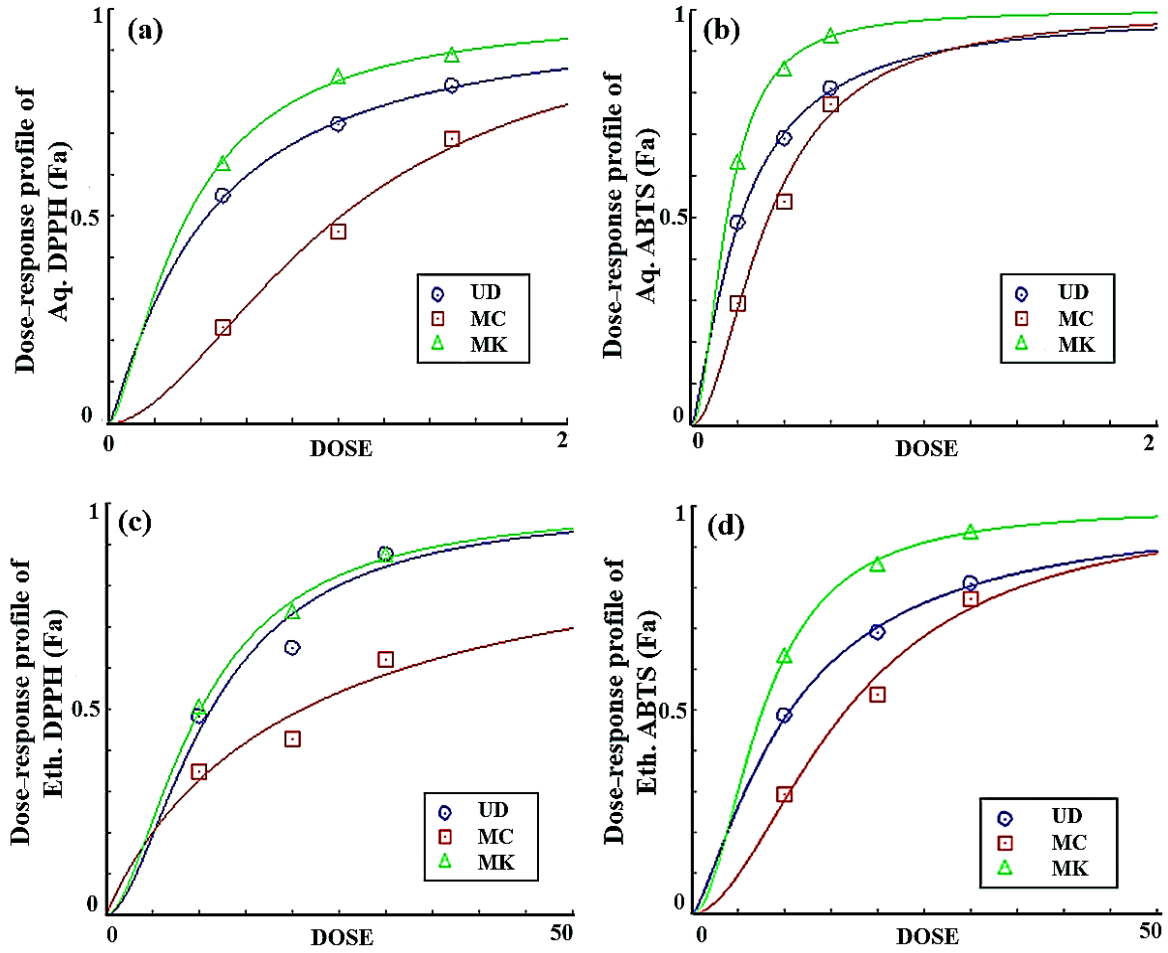


Figure 6.2: Dose-response profile of the estimated EC_{50} (mg/ml) of aqueous (a and c) and ethanolic (b and d) extracts of *U. dioica* (UD), *M. chamomilla* (MC), and *M. koenigii* (MK) in the DPPH (a and b) and ABTS (c and d) assays.

The leaves of MK were found to have higher antioxidant capacity due to the existence of greater total phenolic and flavonoid contents, with EC_{50} values of 0.33 mg/ml and 0.10 mg/ml for aqueous and ethanolic extraction of DPPH scavenging and 0.51 mg/ml and 0.07 mg/ml for aqueous and ethanolic extraction of ABTS radicals, respectively, than UD leaves and MC flowers (**Figure 6.2)**.

6.4 Phenolic and flavonoid potential of UD, MC, and MK extracts

TPC Total Phenolic Content and TFC Total Flavonoid Content are commonly used assays to determine the concentration of phenolic compounds and flavonoids, respectively, in plant extracts. These assays provided the valuable information about the antioxidant and potential health-promoting properties of plant materials. Individual aqueous and ethanolic extracts of UD, MC, and MK were quantified for total phenolic and flavonoid content.

The TPC assay measures the overall concentration of phenolic compounds in a plant extract and calculated on the basis of gallic acid standard curve. In terms of aqueous extracts, MK (35.14 mg (GAE)/g) was found to be highest, followed by UD (26.08 mg (GAE)/g) and lowest in MC (24.01 mg (GAE)/g). The trend was the same in the case of ethanolic extracts: MK (48.93 mg (GAE)/g) followed by UD (42.93 mg (GAE)/g) and lowest in MC (40.5 mg (GAE)/g), as shown in **Figure 6.3**.

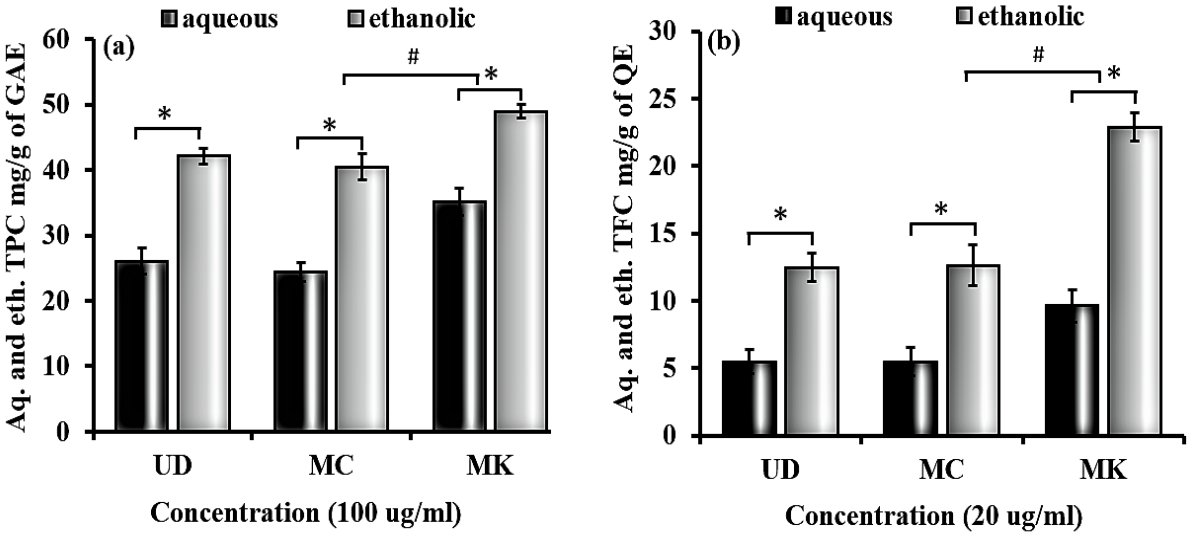


Figure 6.3: Total phenolic (a) and flavonoid (b) contents of aqueous and ethanolic fractions of *U. dioica, M. chamomilla* and *M. koenigii.* Data are shown as the mean ± SD for n = 3. Statistical significance is ascribed as **P*<0.05 (intra group) and #*P*<0.05 (inter group) of the respective groups.

The TFC assay measures the overall concentration of flavonoids in a plant extract and calculated on the basis of quercetin standard curve. It was highest in MK leaves (9.64 mg (QE)/g), followed by MC flowers (5.46 mg (QE)/g) and lowest in UD leaves (5.45 mg (QE)/g) in the case of aqueous extracts, and the trend was the same in the case of ethanolic extracts: MK (22.88 mg (GAE)/g) followed by MC (12.64 mg (GAE)/g) and lowest in UD (12.48 mg (GAE)/g), as shown in **Figure 3**.

The amount of total phenolic and flavonoid in the aqueous fractions of UD, MC, and MK was lower than that of ethanolic extracts. This might be due to the aqueous solvent and extraction process employed. It was also shown that the solvent employed for extraction might be to blame for the quantity of phenolic content in the extract. MK had the highest TPC and TFC levels than UD and MC in both aqueous and ethanolic extracts. The presence of hydroxyl groups in phenols allows them to scavenge free radicals, proving that they are the most essential phytochemicals.

6.5 The correlation study between total phenolic or flavonoid content and EC_{50} of antioxidant activity of plant extracts

Phenolic compounds and flavonoids are known to possess antioxidant properties and play a significant role in scavenging free radicals and reducing oxidative stress. A correlation between phenolic and flavonoid with EC_{50} of antioxidant assays (DPPH and ABTS) was expressed with Pearson's correlation coefficient (r). This study revealed that, MK extract demonstrated a strong positive correlation coefficient between phenolic and flavonoid with EC_{50} of antioxidant assays (DPPH and ABTS). UD extract also showed a high positive correlation coefficient between phenolic and flavonoid with EC_{50} of antioxidant assays (DPPH and ABTS). MC extract revealed a moderate correlation coefficient between phenolic and flavonoid with EC_{50} of antioxidant assays (DPPH and ABTS).

The coefficient correlation between antioxidant capacity and flavonoid as well as phenolic concentrations were incredibly high in all extracts (aqueous and ethanolic). As a result, the flavonoid as well as phenolic components of plants considerably contribute to their antioxidant properties. A strong positive correlation was observed between EC_{50} values and phenolic content of plant extracts (**Table 6.2).** It implies that higher amounts of phenolic or flavonoid chemicals are related with enhanced antioxidant activity. This finding suggest that the plant extract has the potential to provide enhanced protection against oxidative damage.

Table 6.2: Evaluation of the coefficient of correlation between antioxidant potential and total phenolic/flavonoid content in plant extracts.

Assays	Plant species	R^2 Value (TPC)	Type of Correlation	R^2 Value (TFC)	Type of Correlation
DPPH (Aqueous)	*U. dioica*	0.890	High	0.878	High
	M. chamomilla	0.619	Moderate	0.819	High
	M. koenigii	0.937	Strong	0.949	Strong
DPPH (Ethanolic)	*U. dioica*	0.918	Strong	0.981	Strong
	M. chamomilla	0.787	High	0.830	High
	M. koenigii	0.967	Strong	0.954	Strong
ABTS (Aqueous)	*U. dioica*	0.837	High	0.772	High
	M. chamomilla	0.633	Moderate	0.633	Moderate
	M. koenigii	0.962	Strong	0.919	Strong
ABTS (Ethanolic)	*U. dioica*	0.914	Strong	0.971	Strong
	M. chamomilla	0.705	High	0.794	High
	M. koenigii	0.999	Strong	0.859	High

"Symbol stands for DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS [(2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)], TPC (Total Phenolic Content), TFC (Total Flavonoid Content). Values between 0.90-1.00 (strongly positive corelation); 0.70-0.90 (high positive correlation); 0.50-0.70 (moderate correlation); 0.30-0.50 (low correlation) and 0.00-0.30 (negligible correlation)" (Hinkle et al., 2003).

6.6 Preliminary qualitative screening analysis of plant extracts

Preliminary qualitative screening analysis of plant extracts involves a set of simple tests or assays to gain initial insights into the presence or absence of certain chemical constituents or bioactive compounds. Phytochemicals are chemical molecules produced by plants as a result of their normal metabolic activities. These substances are referred to as secondary metabolites. Phytoconstituents such as amino acids, polyphenols, reducing sugars, alkaloids, terpenoids, glycosides, carbohydrates, and saponins are mostly responsible for curative capabilities such as menstruation problems, muscular spasms, anemia, ulcers, hemorrhoids, inflammation, and wound healing. To confirm the existence of phytoconstituent substances, a phytochemical screening study was performed on the crude extracts of UD, MC, and MK along with appropriate chemical tests.

The presence of bioactive phytochemical substances such as phenols, flavonoids, alkaloids, tannins, carbohydrates, saponins, terpenoids, steroids, and glycosides are represented in **Table 6.3**.

Table 6.3: Preliminary qualitative screening of secondary metabolites of crude extracts of UD, MC, and MK.

S. No.	Phytoconstituents	Tests	Positive results	UD	MC	MK
1.	Phenols	Ferric chloride test	Bluish-green color	+	+	+
2.	Flavonoids	Alkaline reagent test	Orange–red	+	+	+
3.	Alkaloids	Wagner's test	Red precipitate	+	+	+
4.	Tannins	$FeCl_3$ test	Coloring black blue	+	+	+
5.	Carbohydrates	Molisch's test	Red or dull violet color	+	-	+
6.	Saponins	Foam test	White precipitates	+	+	+
7.	Terpenoids	Salkowski test	Change color pink to violet	+	+	+
8.	Steroids	Liebermann's test	Violet to bluish-green color	+	+	+
9.	Glycosides	Keller-Killiani test	Coloring brick red	-	+	+

Note: The presence of phytoconstituents is indicated by a '+' sign, whereas the lack of phyto-constituents is indicated by '-'.

6.7 UV–Visible analysis of plant extracts

Spectral data were observed in the range of 200-800 with intervals of 1 nm and showed maximum absorption at 284 and 321 nm in the case of UD, 267 and 326 nm for MC, and 286 and 317 nm for MK **(Figure 6.4)**. With increasing concentration, the absorption increased. This technique showed the quantitative analysis of UD, MC, and MK concerned with the absorption of radiation in the ultraviolet and visible spectra. This radiation enables electrons in atoms or molecules to shift from lower to higher energy levels. Under regulated conditions, the amount of radiation absorbed is proportional to the intensity of chemicals in the plant extracts was observed. Spectral analysis showed peaks in crude extracts of UD, MC, and MK indicating the presence of a variety of ingredients/chemicals and particularly providing information about unsaturated bonds in conjugated or aromatic components. For example, various pigments, such as chlorophylls and carotenoids, present in plant extracts may exhibit distinctive absorption peaks in the visible region of the spectrum.

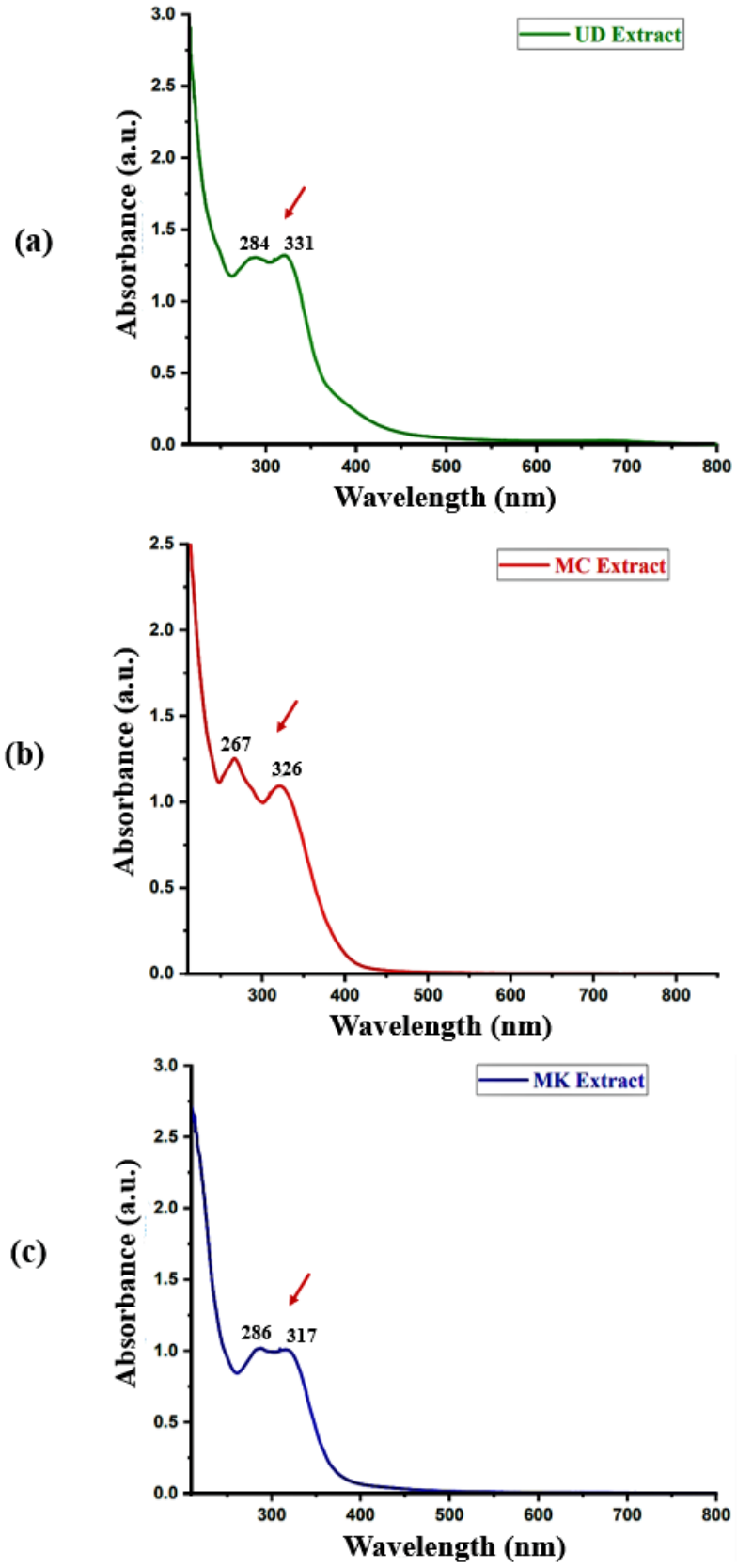


Figure 6.4: UV-Visible spectra of *U. dioica* (a), *M. chamomilla* (b), and M. koenigii (c) fractions. Red arrows represent the peak of extract (conjugated or chemical bonds).

6.8 Fourier Transform Infrared Spectrophotometer (FTIR)

The functional groups of bioactive components present in plant extracts of UD, MC, and MK were identified using the FT-IR spectra depending on peak values in the IR radiation area. When the extract was run through FTIR, the functional groups of the constituents were segregated based on the ratio of their peaks. The existence of alkanes, amino acids, aldehydes, phenols, secondary alcohols, aromatic amines, ketones, and halogen compounds was verified by FTIR analysis **(Figure 6.5)**.

In the ethanolic extracts of UD, MC, and MK, the wideband between 3500 cm^{-1} and 3200 cm^{-1} was assigned to an O-H (stretch), indicating that hydroxyl and phenolic groups are present. These groups can be found in cellulose, hemicellulose, and lignin structures. These groups are related to the extract's hydroxylated compounds (polyphenols) as well as the plant extract's moisture content. A minor intensity peak in the region of 2950-2960 cm^{-1} shows an O–H (stretch), which may suggest the existence of amide groups and alcohols as well as C–H vibrations of the CH_3 group. In the area of 1650-1600 cm^{-1}, an acceptable N=O (stretch) vibration is recorded. Furthermore, weak peaks in this area should suggest the existence of C=C (stretch). Nevertheless, the existence of carbonyl groups may be indicated by absorption C=C (stretch). Nevertheless, the existence of carbonyl groups may be indicated by absorption vibration in this region. A doublet band is seen in the fingerprint area between 1455 cm^{-1} and 1370 cm^{-1} indicating the C-H (bending) vibration of the methyl group ($–CH_3$) molecule. Furthermore, a broad peak was found between 1100 cm^{-1} and 1000 cm^{-1}, showing the presence of alcohol groups within the compound structure. This is most likely due to an alkoxy C–O (stretching) vibration. In addition, the UD, MC, and MK fractions showed aromatic C–H bonds between 720 and 620 cm^{-1} in the infra-red spectrum **(Table 6.4)**.

The FTIR analysis of plant extracts revealed information on the plant's chemical constituents, such as organic compounds, functional groups, and other biomolecules. Carbohydrates, such as hemicellulose, cellulose, and different sugars, are present. Carbohydrates usually have distinct absorption bands in the fingerprint area (1500-500 cm^{-1}). Plant extracts include proteins, which may be identified in the mid-infrared area via amide bands. Amide I (C=O stretching) and Amide II (N-H bending) bands are frequently found in protein FTIR spectra. The distinctive absorption bands of lipids contained in plant extracts, such as fatty acids and triglycerides, are 3000-2800 cm^{-1} (stretching vibrations of C-H bonds) and about 1740 cm^{-1} (C=O stretching).

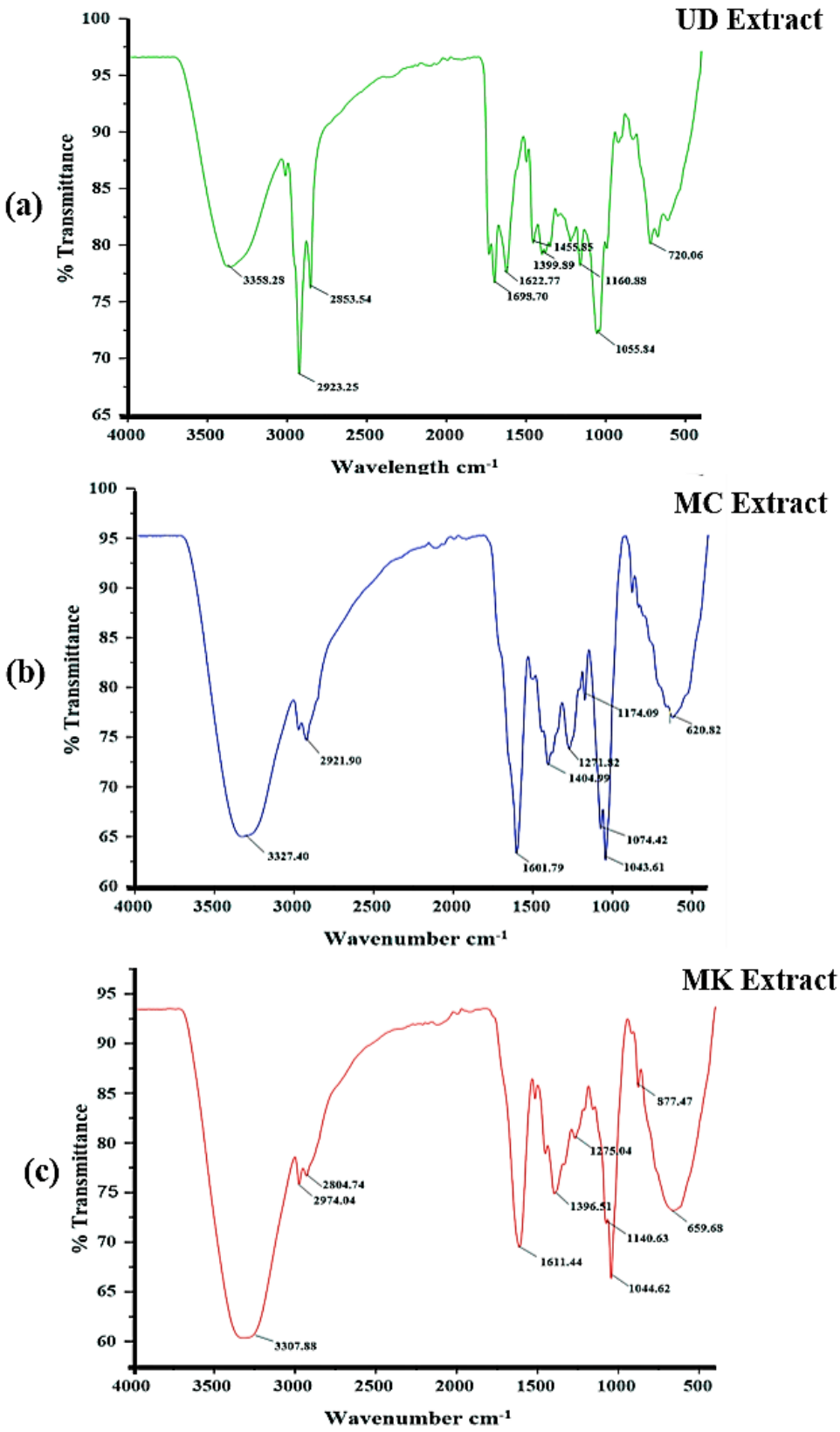


Figure 6.5: The FT-IR absorption spectrum of UD (a), MC (b), and MK (c) extracts with a scan range of 400-4000 cm^{-1}.

Table 6.4: FT-IR frequency range and functional groups present in the extracts of UD, MC, and MK.

S. no.	Frequency range (cm^{-1})			Functional groups	Phytocompounds identified
	UD	MC	MK		
1.	3358.28	3327.40	3307.88	H-bonded, OH stretching	Hydroxyl compounds
2.	2923.25	2921.90	2974.04	Asymmetric stretching –CH(CH_2) vibration	Saturated aliphatic Compound (Lipids)
3.	2853.54	2802.86	2804.74	Symmetric stretching –CH_2(CH_2) vibration	Proteins, lipids
4.	1698.70, 1622.77	1601.79	1611.44	C=O stretching vibration	Ketone compound
5.	1455.85	1404.99	-	C=C-C Aromatic ring stretching	Aromatic compound
6.	1399.89	-	1396.51	O-H, alcoholic group	Phenol or tertiary alcohol
7.	-	1271.82	1275.04	CN stretching	Aromatic primary amine
8.	1160.88	1174.09	1140.63	Polymeric OH, C-O stretching	Cyclic ether
9.	1055.84	1074.42, 1043.61	1044.62	Phosphate ion	Phosphate compound
10.	-	-	877.47	P-O-C stretching	Aromatic phosphate
11.	720.06	620.82	659.68	C-Cl stretching	Aliphatic chloro compound

Note: The absence of energy bands is indicated by '-'.

Finally, the results revealed that the chemical structures of UD, MC, and MK are extremely polar (lignin (+)-neoolivil, 3,4-divanillyltetrahydrofuran, isolariciresinol, pinoresinol, and (-)-secoisolariciresinol), which stimulates the proliferation of human lymphocytes and has anti-inflammatory effects, as evidenced by the presence of wide peaks within the spectra.

6.9 High performance liquid chromatography (HPLC)

The external standard technique was used to perform HPLC experiments under isocratic conditions. The ethanolic extracts of UD, MC, and MK were analyzed directly on the total extracts without any manipulation. The retention time of the chromatographic peaks of plant extracts were compared to those of reference standards (rutin and quercetin), and DAD spectra (200–400 nm) to analyze them **(Figure 6.6)**. The findings of this study revealed the presence of 25 compounds (**Table 6.5)** in the ethanolic extracts of UD, MC, and MK, including quercetin, coumaric acid, chlorogenic acid, gallic acid, apigenin, myricetin, ferulic acid, fumaric acid, rutin, isorhamnetin, kaempferol, etc.

In the ethanolic extract of UD, three classes of phenols were characterized: anthocyanin compounds (rosinidin 3-O-rutinoside; peonidin 3-O-rutinoside and peonidin 3-O-(6′-O-p-coumaroyl glucoside)**,** hydroxycinnamic acid derivatives (p-coumaric acid; chlorogenic acid; caffeoylquinic acid; 2-O-caffeoylmalic acid), and flavonoids (rutin; isorhamnetin 3-O-rutinoside; kaempferol 3-O-glucoside; quercetin; p-coumaroyl glucoside; kaempferol 3-O-rutinoside and quercetin 3-O-glucoside) were observed.

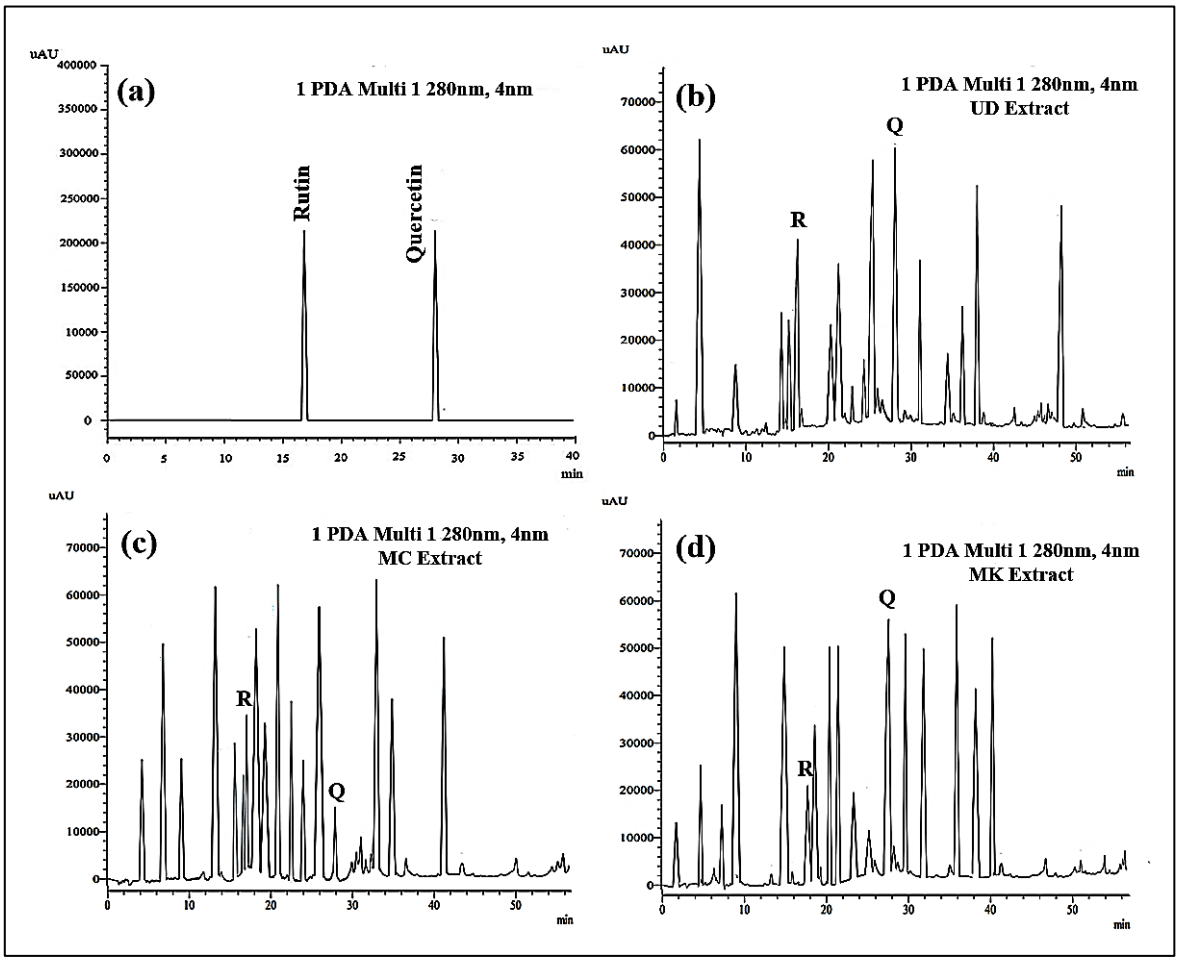
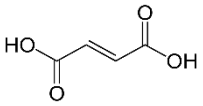
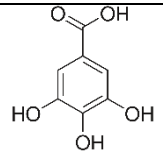
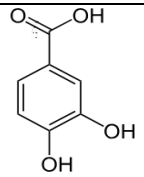
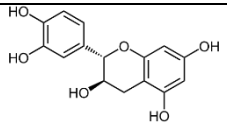
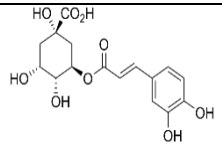
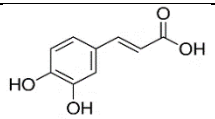
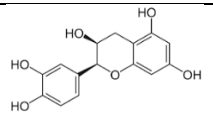
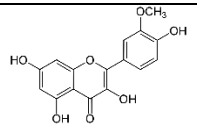
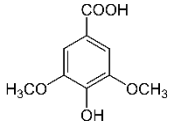
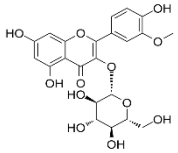
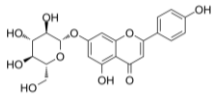
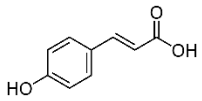


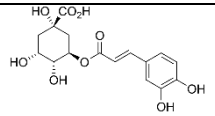
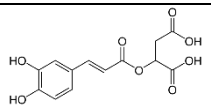
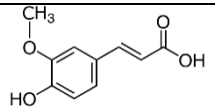
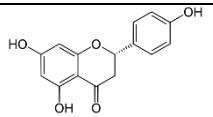
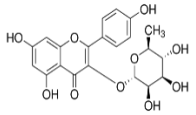
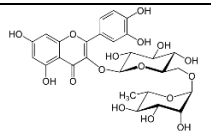
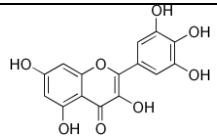
Figure 6.6: HPLC profiles acquired at 280 nm of standard rutin and quercetin (a) and ethanolic extracts obtained from *U. dioica* leaves (b), *M. chamomilla* flowers (c), and *M. koenigii* leaves (d) sowing different bioactive compounds.

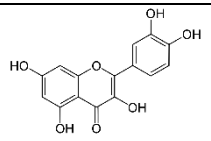
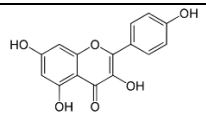
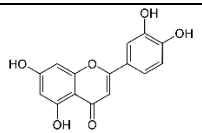
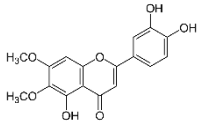
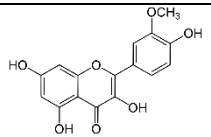
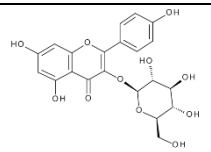
The polyphenolic compounds found in the ethanolic fractions of MC flowers were identified as an essential constituents such as quercetin (quercetin-7-O-β-glucoside; quercetin-3-O-β-rutinoside and quercetin-3-O-β-galactoside), apigenin (apigenin-7-O-7-glucoside; apigenin-7-O-apiosyl-glucoside and apigenin-7-O-glucosyl-6'-acetate), luteolin (luteolin-7-O-β-glucoside; luteolin-4'-O-7-β-glucoside and luteolin-7-O-β-rutinoside), isorhamnetin (isorhamnetin-7-O-β-glucoside), patuletin (patuletin-7-O-β-glucoside), eupatoletin, astragalin, chrysosplenol and spinacetin.

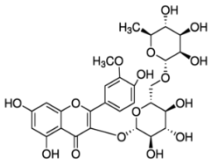
Table 6.5: Major phytochemical compounds identified in ethanolic extracts of *U. dioica, M. chamomilla,* and *M. koenigii.*

S. No	Retention time (R_t min.)			Compound	Molecular formulae	Chemical Structure	Molecular weight (g/mol)	Pharmacological actions
	UD	MC	MK					
1.	1.931	-	1.645	Fumaric acid	$C_4H_4O_4$		116.07	Reduce gallstone formation, used for the treatment of multiple sclerosis and psoriasis.
2.	4.240	4.235	4.276	Gallic acid	$C_7H_6O_5$		170.12	Expectorant, cytotoxic steroid, memory enhancer, anti-inflammatory, anti-neoplastic, and antioxidant properties.
3.	-	7.523	7.473	Protocatechuic acid	$C_7H_6O_4$		154.12	Neuroprotective, antioxidant, anticancer, antibacterial, anti-aging, and anti-asthma properties.
3.	9.211	-	9.865	Catechins	$C_{15}H_{14}O_6$		290.27	To prevent and treat various diseases, high antioxidant activity and used in cosmetics.
4.	-	11.387	-	4-O-Caffeoylquinic acid	$C_{16}H_{18}O_9$		354.31	Cytoprotective, neuroprotective, and hepatoprotective effects.

5.	15.982	14.683	15.554	Caffeic acid derivative	$C_9H_8O_4$		180.16	Prevents DNA damage and oxidative stress induced by free radicals.
6.	16.683	16.299	16.693	Epicatechin	$C_{15}H_{14}O_6$		290.27	Reduce blood glucose levels in diabetic patients and stimulate mitochondrial respiration.
7.	17.248	17.558	17.280	Isorhamnetin-7-O-glucoside	$C_{22}H_{22}O_{12}$		478.4	Anti-coagulant, anti-inflammatory, and anti-proliferative properties.
8.	17.515	17.428	17.625	Syringic acid	$C_9H_{10}O_5$		198.17	Therapeutic uses in prevention of CVDs, cancer, diabetes, and possess antioxidant activities.
9.	18.094	18.763	18.651	Isorhamnetin-3-O-glucoside	$C_{22}H_{22}O_{12}$		478.4	Anti-viral, antioxidant, anticancer, Antitumor, anti-inflammatory, and antimicrobial properties.
10.	-	19.243	-	Apigenin-7-O-glucoside	$C_{21}H_{20}O_{10}$		432.4	Prominent chemopreventive, anti-candidal effect, antifungal potential and strengthen the failing heart.
11.	20.728	20.152	20.835	p-coumaric acid	$C_9H_8O_3$		164.16	Anti-inflammatory, antimicrobial, anti-viral, and antibacterial.

12.	-	21.739	-	4,5-O-dicaffeoylquinic acid	$C_{25}H_{24}O_{12}$		516.4	In melanocytes, it significantly reduces tyrosinase activity and melanin synthesis in a dose-dependent manner.
13.	22.564	22.571	22.677	2-O-Caffeoylmalic acid	$C_{13}H_{12}O_8$		296.230	Prevents ROS production and possesses high antioxidant activity.
14.	23.232	23.924	23.232	Ferulic acid	$C_{10}H_{10}O_4$		194.18	Wide range of therapeutic uses against various diseases including cancer, arthritis, etc.
15.	-	24.579	-	Naringin	$C_{27}H_{32}O_{14}$		580.5	Anti-carcinogenic and act as inhibition of selected cytochrome P_{450} enzymes.
16.	25.247	25.460	25.814	Quercetin (quercetin-3-O-rhamonoside)	$C_{21}H_{20}O_{11}$		448.4	Used for the treatment of inflammatory, allergic, metabolic disorders and act as anti-protozoal.
17.	26.024	26.963	26.165	Rutin	$C_{27}H_{30}O_{16}$		610.5	Hypolipidemic, anti-protozoal, vasoactive, cytoprotective, anti-allergic, anti-platelet, anti-hypertensive and anti-spasmodic.
18.	27.723	27.787	27.379	Myricetin	$C_{15}H_{10}O_8$		318.23	Act as anti-epileptic, anti-amyloidogenic, anti-diabetic, antioxidant, antibacterial, anti-ulcer, antiviral, anticancer, and anti-inflammatory.

19.	28.231	28.789	29.031	Quercetin	$C_{21}H_{20}O_{11}$		448.4	It decreases tumor necrosis factor (TNF-α) production in macrophages and LPS-driven IL-8 synthesis in lung A549 cells generated by lipopolysaccharide (LPS).
20.	32.156	32.956	31.456	Kaempferol	$C_{15}H_{10}O_6$		286.24	Anxiolytic, anti-diabetic, anti-estrogenic, anti-osteoporotic, cardioprotective, and neuroprotective.
21.	-	34.320	-	Luteolin	$C_{15}H_{10}O_6$		286.24	It exhibits anti-inflammatory properties due to its ability to regulate transcription factors like NF-B, AP-1, and STAT3.
22.	-	34.745	-	Cirsiliol	$C_{17}H_{14}O_7$		330.29	Act as inhibitors of arachidonate 5-lipoxygenase and has anticancer, hypnotic, sedative, and anti-inflammatory properties.
23.	35.339	35.445	36.021	Isorhamnetin	$C_{16}H_{12}O_7$		316.26	Cerebrovascular and cardiovascular protective properties, in addition, it also acts as antioxidant, antitumor, anti-inflammatory, organ protection, and obesity prevention properties.
24.	37.277	-	37.552	Kaempferol 3-O-glucoside	$C_{21}H_{14}O_{11}$		448.38	It lowers the risk of chronic diseases, particularly cancer, and boosts the body's antioxidant defenses against free radicals.

25.	39.901	41.026	40.516	Isorhamnetin 3-O-rutinoside	$C_{28}H_{32}O_{16}$		624.5	Inhibit membrane protein, anti-apoptosis antioxidation, antitumor anti-inflammation, anti-viral and antibacterial, anti-amyloidogenic, and anti-diabetic properties.

Note: The absence of compounds is indicated by '-'.

The MK ethanolic fraction was examined by HPLC-DAD, which permitted the identification of important components such as chlorogenic acid, quercitrin, citric acid, piperine, 7 p-coumaric acid, hesperidin, rutin, gallic acid, β-terpineol, ferulic acid, catechin, naringenin, D-α-pinene, di-α-phellandrene, dipentene, D-sabinene, caryophyllene, nicotinic acid, koenigine-quinone A and koenigine-quinone B.

All these secondary metabolites have been shown to have cerebrovascular protective, neuroprotective, and cardiovascular protective properties. In addition, it also acts as an anti-carcinogenic, antitumor, anti-inflammatory, anti-microbial, anti-viral, anti-bacterial agent and protects against oxidative stress‑related diseases.

Rutin and quercetin were used as standard compounds in HPLC analysis of plant extracts due to their wide presence in various plant sources and their well-established chromatographic behavior. They belong to a class of compounds known as flavonoids, which are widely distributed in fruits, vegetables, and other plant materials.

To identify and quantify the components contained in the plant extract, the retention time, peak area, and peak height of each compound was analysed. The compounds detected in the HPLC profile for plant extracts (UD; MC; and MK) can vary widely, and they may include phenolic compounds (such as phenolic acids and flavonoids), terpenoids, alkaloids, glycosides, and other secondary metabolites. The precise chemicals present, as well as their relative amounts, will vary depending on the plant species and its natural chemical composition.

6.10 Synthesis of green engineered ZnO nanoparticles using UD, MC, and MK extracts

The green fabrication mechanism for the generation of ZnO nanoparticles based on plant phytochemicals that can function as stabilizing and capping compounds to con-vert metal salts to metal nanoparticles. The major phytochemicals including methylxanthines, alkaloids, aldehydes, terpenoids, flavonoids, and phenolic acids served as a stabilizing agent that prevented particle agglomeration. These phytoconstituents (capping/reducing agents) are present in various plant extracts at varying concentrations. Thus, the synthesis of NPs is considerably influenced by the composition of plant fraction. Zinc (II) ions in plant extract are converted to metallic zinc, in-stead of generating a coordinated complex. Following the full reduction of the zinc precursor, metallic zinc and the dissolved oxygen in the solution reacted, resulting in the generation of Zinc oxide nuclei.

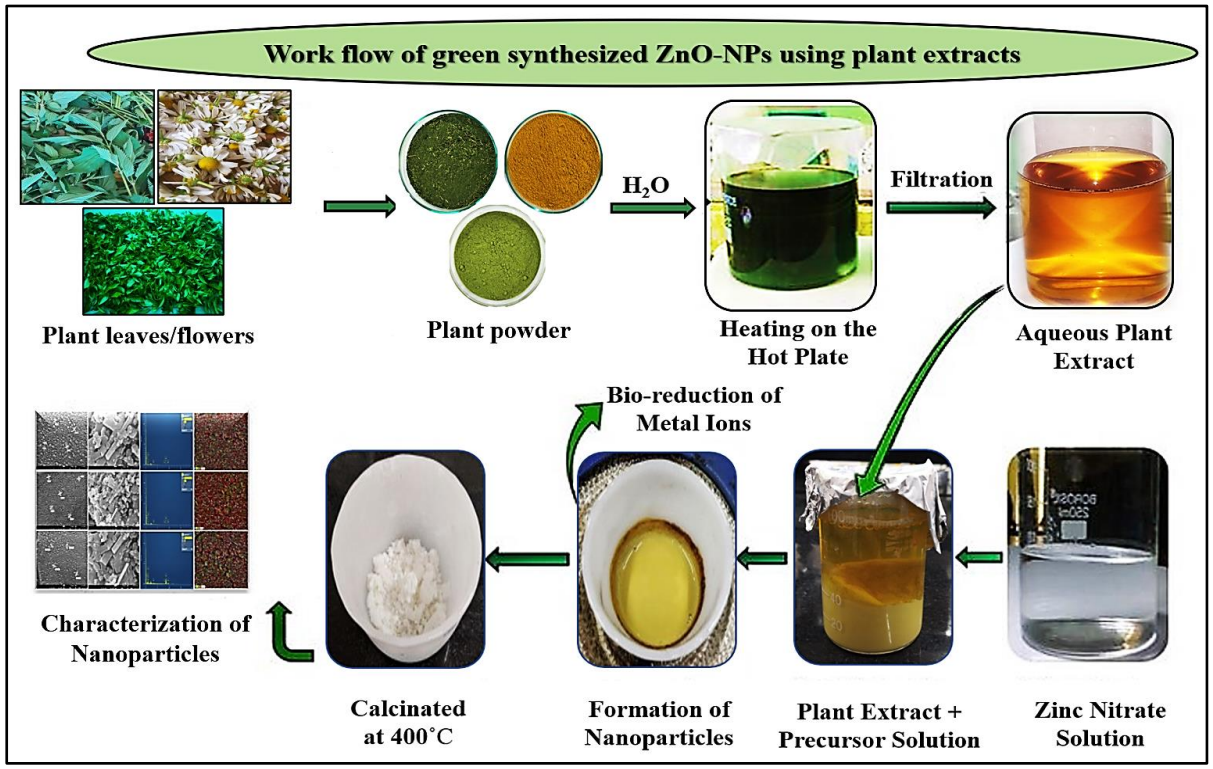


Figure 6.7. Plant-mediated process of biogenic ZnO nanoparticles include the extraction of plant fraction, combining of the metal precursor solution in the plant fraction, and generation of green engineered NPs. The NPs were then analyzed using techniques like FTIR, SEM, XRD, EDX, and UV-Visible.

The fabrication, stability, and generation of NPs are all dependent on factors including pH, metal salt content, contact time, temperature, and phytochemical composition of plant fraction. In order to stabilize the metal ions after being reduced by plant extracts, they will be encapsulated as an organic coating in three processes. Metal ion reduction and the nucleation

of reduced metal ions are involved in the activation phase **(Figure 6.7)**.The growth phase contributes to the stability of nanoparticles. The NPs shapes are determined in the termination phase.

6.11 Characterization of biosynthesized zinc oxide nanoparticle (ZnO-NPs)

Characterization is a fundamental process for determining the size, morphology, shape, size distribution, composition, surface area, and surface charge of the green fabricated ZnO-NPs. The techniques that have been employed to characterize ZnO-NPs are listed below.

6.11.1 Optical properties of ZnO-NPs using UV-Vis spectroscopy

The optical attributes of synthesized ZnO nanoparticles were assessed utilizing ultra-violet and visible absorption spectroscopy. The absorbance spectra between 200-800 nm were employed to record the biosynthesis of UD-ZnO, MC-ZnO, and MK-ZnO NPs in an aqueous solution.

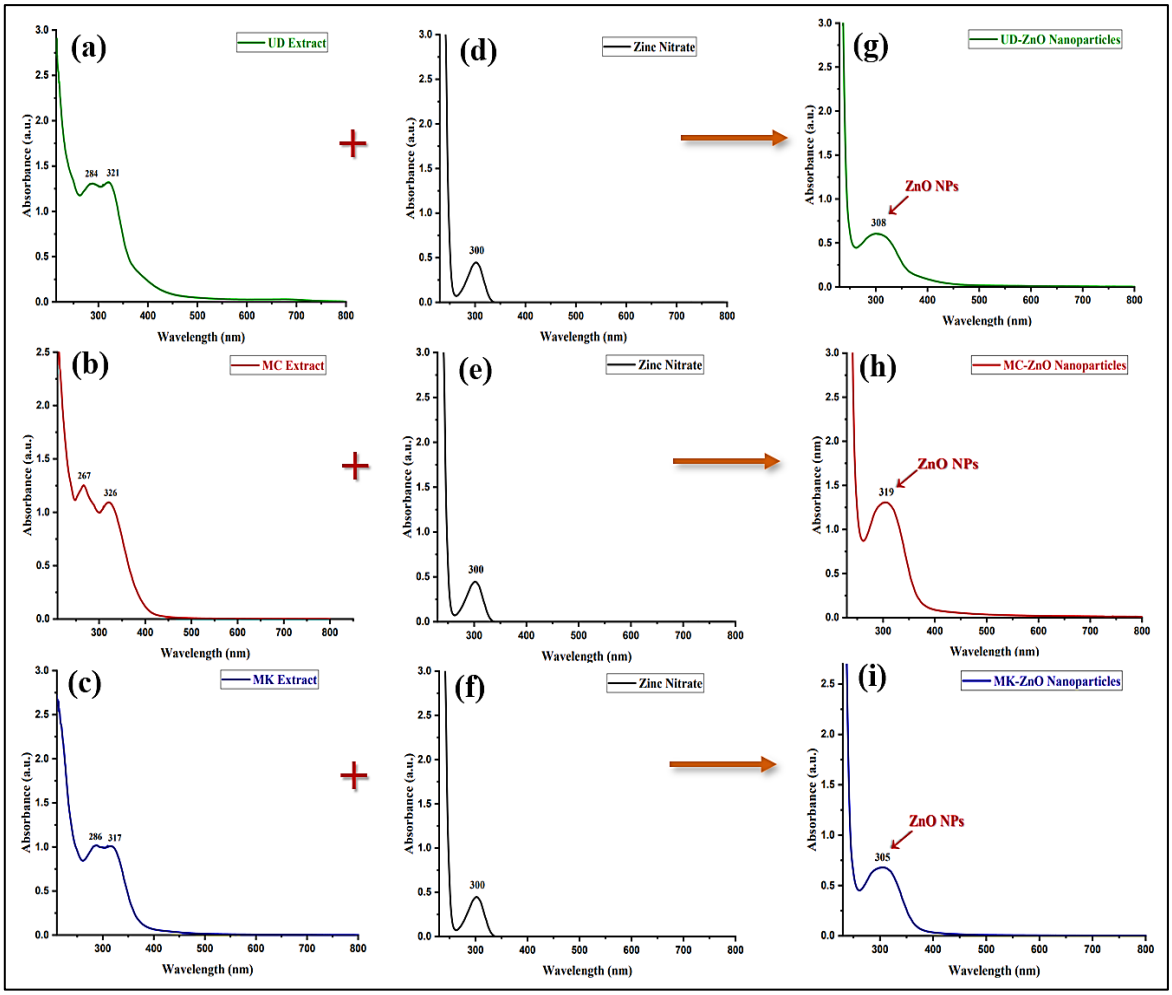


Figure 6.8: UV–Visible spectra of zinc nitrate, *U. dioica* (UD), *M. chamomilla* (MC), and *M. koenigii* (MK) extracts and their respective phytofabricated ZnO-NPs.

The absorption peaks for UD-ZnO, MC-ZnO, and MK-ZnO NPs were obtained at wavelengths of 308, 319, and 305 nm, respectively **(Figure 6.8)**. The absorption edge consistently moves to a higher energy or lower wavelength when the size of the NPs decreases. Earlier studies have identified the peaks at 289-385 nm as the SPR (surface plasmon resonance) of ZnO nanoparticles. Using the formula below, the energy band gap (Eg) is calculated from the UV-Vis graphs of UD-ZnO, MC-ZnO, and MK-ZnO NPs as 4.0, 3.8, and 4.0eV, respectively.

$$Eg = 1240 / \lambda_{max} eV$$

The findings showed that ZnO-NPs were successfully synthesized using aqueous extracts of UD (leaves), MC (flowers), and MK (leaves).

6.11.2 Crystallographic analysis of ZnO-NPs using X-ray Diffraction

Crystallographic analysis of plant-synthesized ZnO-NPs using X-ray diffraction is a significant technique for investigating the phase composition and crystal structure of the nanoparticles. XRD offers information on the arrangement of atoms inside the crystal lattice, allowing crystallographic characteristics such as lattice constants, crystallite size, and crystal structure to be determined. ZnO-NPs were subjected to intense XRD rays during the analysis, and these rays pass through the material to reveal information about its physical properties, chemical composition, and crystallographic structure. The diffraction peaks were obtained for 2θ values at 31.7, 34.5, 36.4, 47.8, 56.2, 62.5, 66.2, 67.6, 69.1, 72.9 and 77.2 corresponds to the plane of reflections for the values of (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202), respectively as depicted in **Figure 6.9**. The results exhibited distinct, strong peaks, which denoted that the biosynthesized ZnO-NPs are pure and crystalline. High intensity peak of ZnO-NPs is identified as the characteristic peak of UD-ZnO, MC-ZnO, and MK-ZnO NPs from the XRD pattern (Figure 4-a,b,c) at 101 planes after comparison with the standard powder diffraction card of JCPDS ‘’Joint Committee on Powder Diffraction Standards’’. The purity of the biogenic . ZnO-NPs is confirmed since no further diffraction peaks were detected.

The average size of the biogenic UD-ZnO, MC-ZnO, and MK-ZnO NPs was 42, 45, and 41 nm, respectively. The particle size decreased when the FWHM value was increased. By performing XRD analysis on plant-synthesized ZnO-NPs, the crystalline properties of ZnO-NPs was observed, which is useful in understanding the chemical and physical characteristics, as well as their prospective applications in diverse domains.

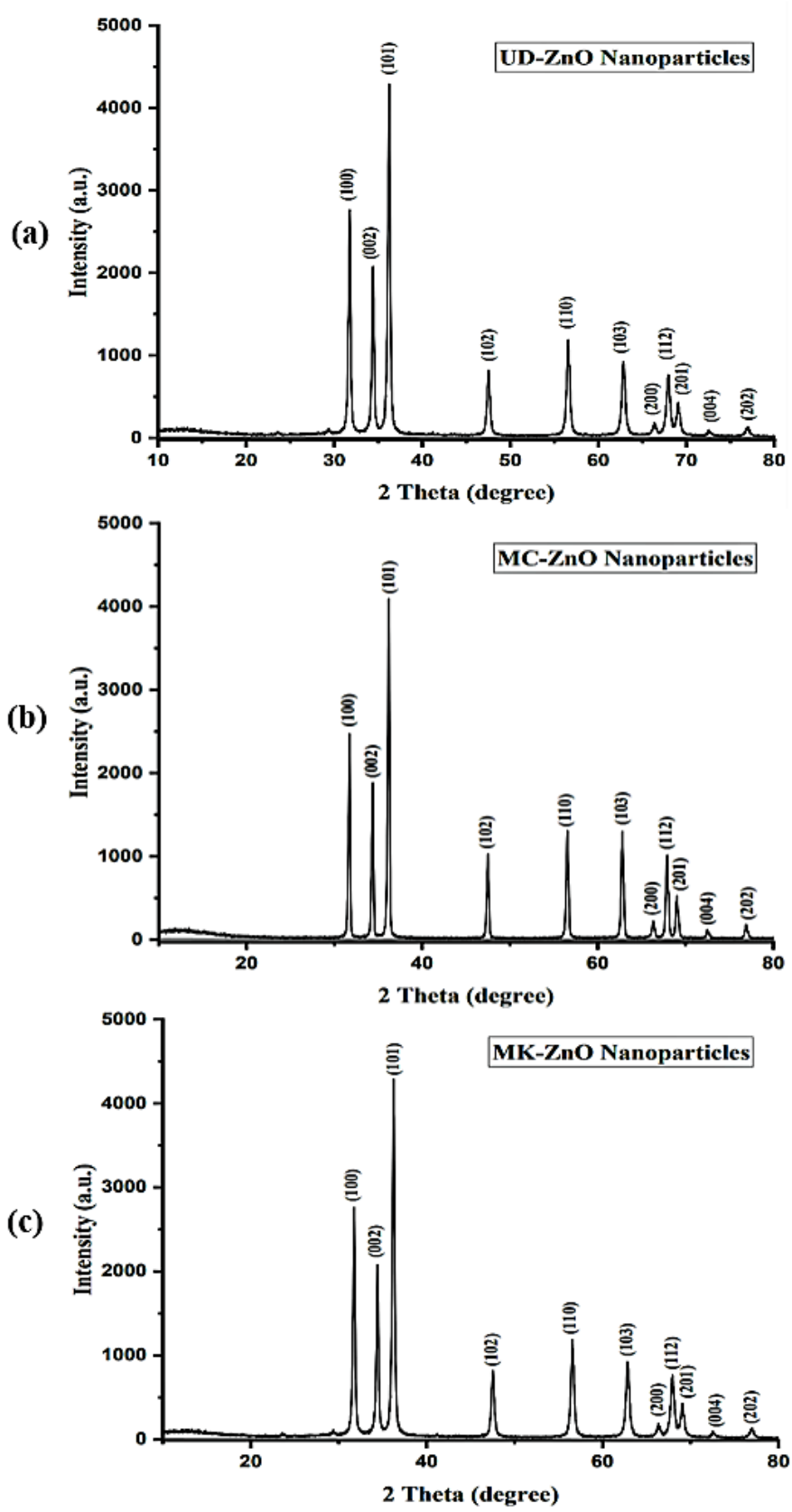


Figure 6.9: The X-ray diffraction of *U. dioica* (a)*, M. chamomilla* (b), and *M. koenigii* (c) ZnO nanoparticles.

6.11.3 Fourier Transform Infrared (FT-IR) analysis of Phyto-fabricated ZnO-NPs

FT-IR spectrum were utilized in the wavelength range of 400-4000 cm^{-1} in order to determine the functional groups present in the fabrication of ZnO nanoparticles from the aqueous fractions of UD (leaves), MC (flowers), and MK (leaves) as illustrated in **Figure 6.10**. The findings of the FTIR study show that the surface of the nanoparticles contains functional groups related to phytoconstituents, namely glycosides, alkaloids, tannins, reducing sugars, ursolic acid, flavonoids, phenols, and others. In this study, the existence of phenolic and hydroxyl groups was indicated by the broad energy bands between 3500-3200 cm^{-1}, which are attributed to stretching of O-H group vibrations. These groups are present in the structures of lignin, cellulose, and hemicellulose. The modest intensity peaks in the 2940-2970 cm^{-1} range that could be assigned to the stretching C-H alkaline vibrations, as well as other possible molecules. The bands observed at 2300-2370 cm^{-1} could be attributed to -C≡C- stretch.

There is a detectable N=O vibration in the range of 1660-1600 cm^{-1}. Additionally, weak peaks in this region should indicate the presence of C=C stretching vibration. However, absorption in this area can be a sign of the presence of carbonyl groups. The 1590-1500 cm^{-1} energy bands might be assigned to C=C/amine-NH stretching. The absorption bands between 1455 and 1370 cm^{-1} can be attributed to aromatic ring C-C stretching. The C-O stretching vibrations of the guaiacyl ring can be attributed to the band between 1260-1200 cm^{-1}. The energy bands identified at 1190-1100 cm^{-1} may correspond to amine stretching. The bands observed between 1096-1010cm^{-1} could be assigned to Si-o-Si stretching protein vibrations. The bands might be corresponded to a secondary amine activity at 867-800 cm^{-1}. The deposition of these substances in the synthesis of ZnO-NPs is indicated by the shift of bands to much lower frequency. In addition, the weak energy bands that were caused by the stretching of the ZnO molecules at 697.28, 542.35, 532.95 cm^{-1} allowed for greater detection of the formation of UD-ZnO, MC-ZnO, and MK-ZnO NPs, respectively. The area that corresponds to metal-oxygen is between 400 and 600 cm^{-1}. The FTIR analysis of plant extracts revealed information on the plant's chemical constituents, such as organic compounds, functional groups, and other biomolecules. Carbohydrates, such as hemicellulose, cellulose, and different sugars, are present. Consequently, it can be concluded that either an oxidation or a reduction mechanism is responsible for the major chemical processes involved in the fabrication of ZnO-NPs utilizing the aqueous extracts of UD, MC, and MK.

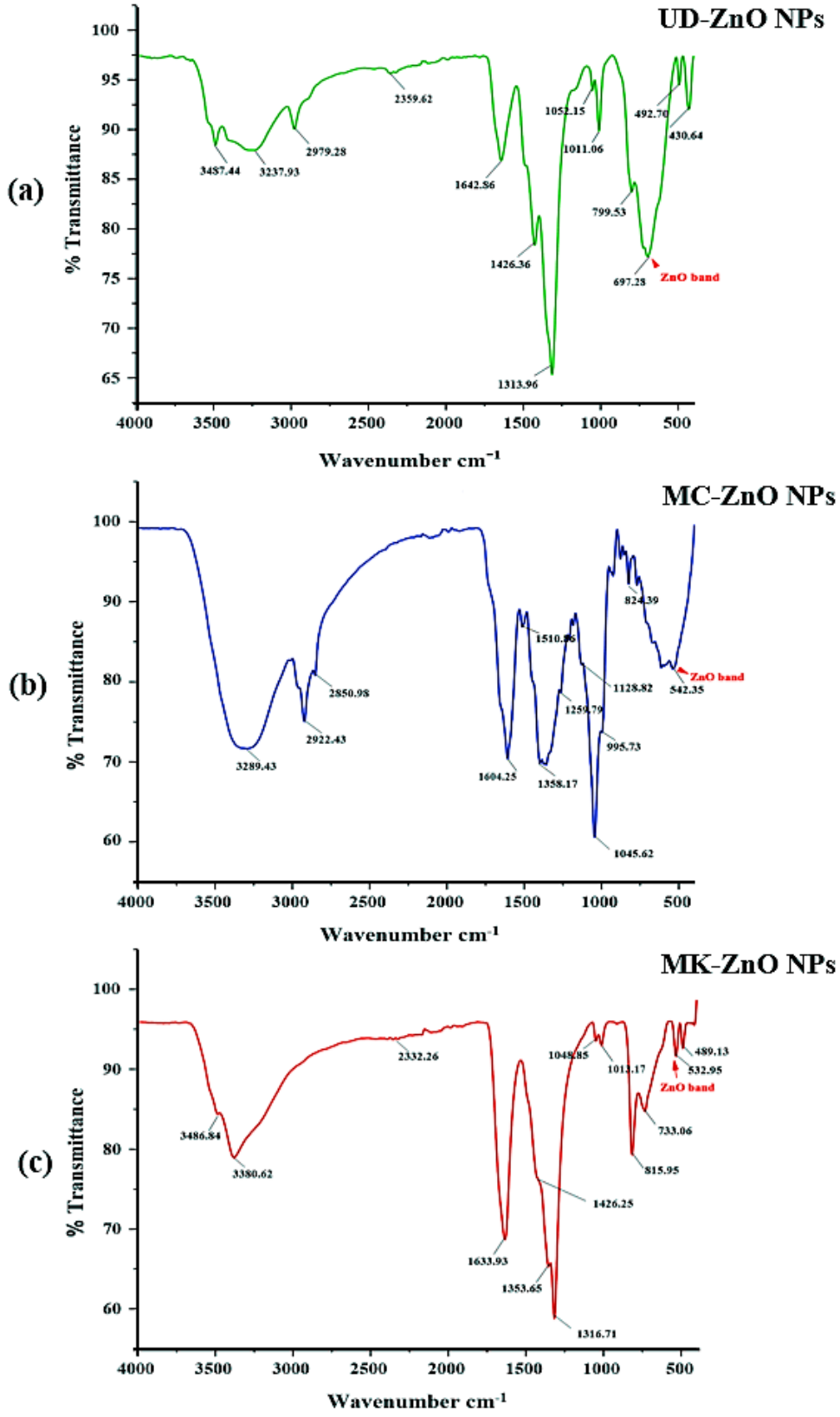


Figure 6.10: The FT-IR absorption spectra with a spectrum range of 400-4000 cm^{-1} of *U. dioica* (a), *M. chamomilla* (b), and *M. koenigii* (c) ZnO nanoparticles.

6.11.4 Field Emission Scanning Electron Microscopy

SEM pictures, depicted that ZnO-NPs were formed in a variety of shapes, some of which were spherical and hexagonal at different temperatures as shown in the SEM images and others of which were irregular. Different magnifications were used to examine the FE-SEM images. The size of UD-ZnO, MC-ZnO, and MK-ZnO NPs, which ranges from 29 to 50 nm, is shown in **Figure 6.11**. The pH, temperature, and zinc nitrate concentration might have all contributed to the uneven morphology of ZnO nanoparticles.

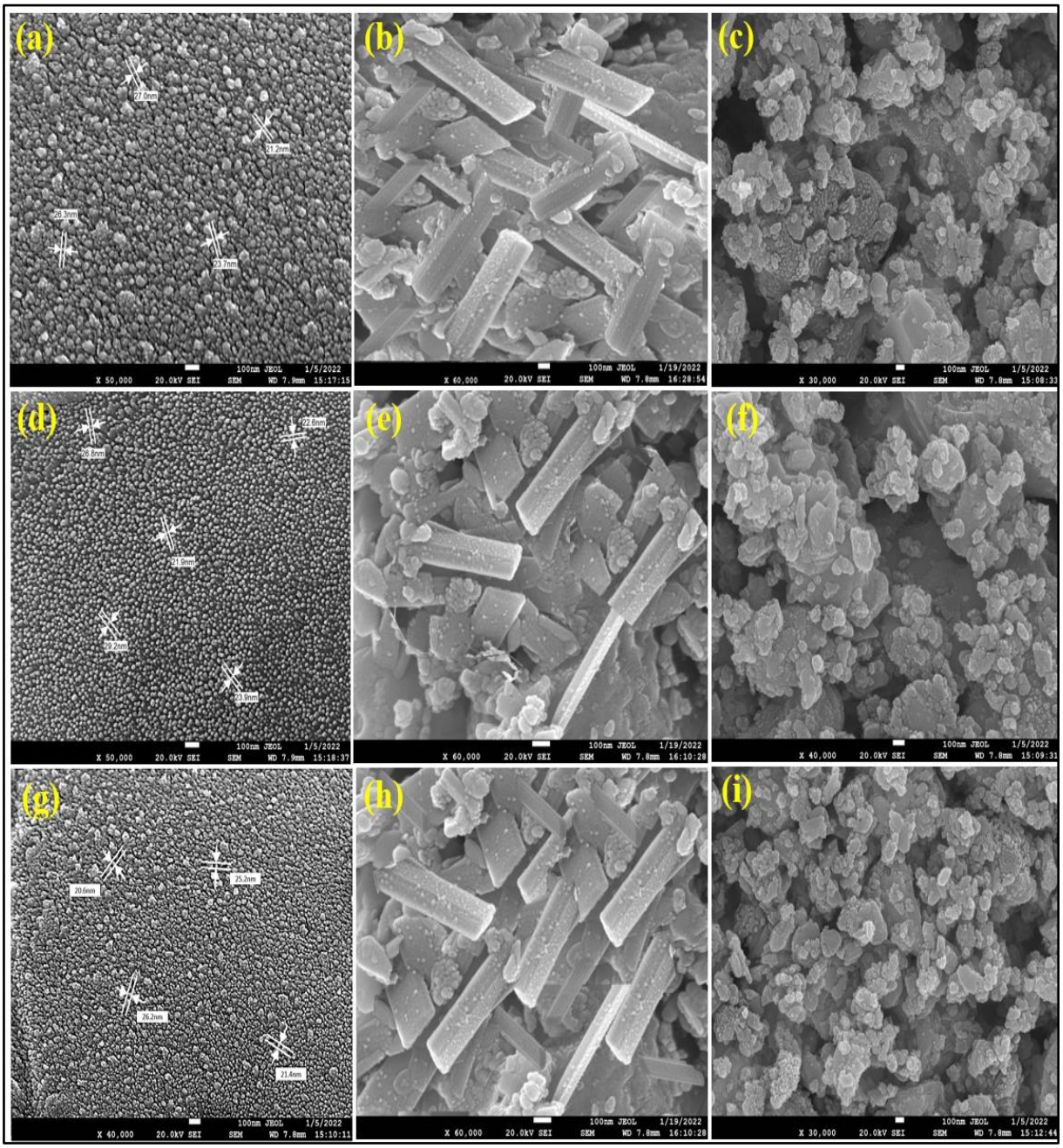


Figure 6.11: FE-SEM images of green synthesized ZnO nanoparticles of *U. dioica* (a,b,c), *M. chamomilla* (d,e,f), and *M. koenigii* (g,h,i) at different magnifications.

6.11.5 Analysis of particle size using zeta potentiometer and dynamic light scattering

The size of the synthesized ZnO-NPs was determined using DLS. In this context, zeta potential is used here to describe the electrochemical charge in the interfacial double layer at the location of the sliding plane in comparison to a site in the bulk fluid far from the interface (**Figure 6.12-a,b,c)**. The stability of a shape or structure is directly correlated with zeta potential, also termed as surface potential.

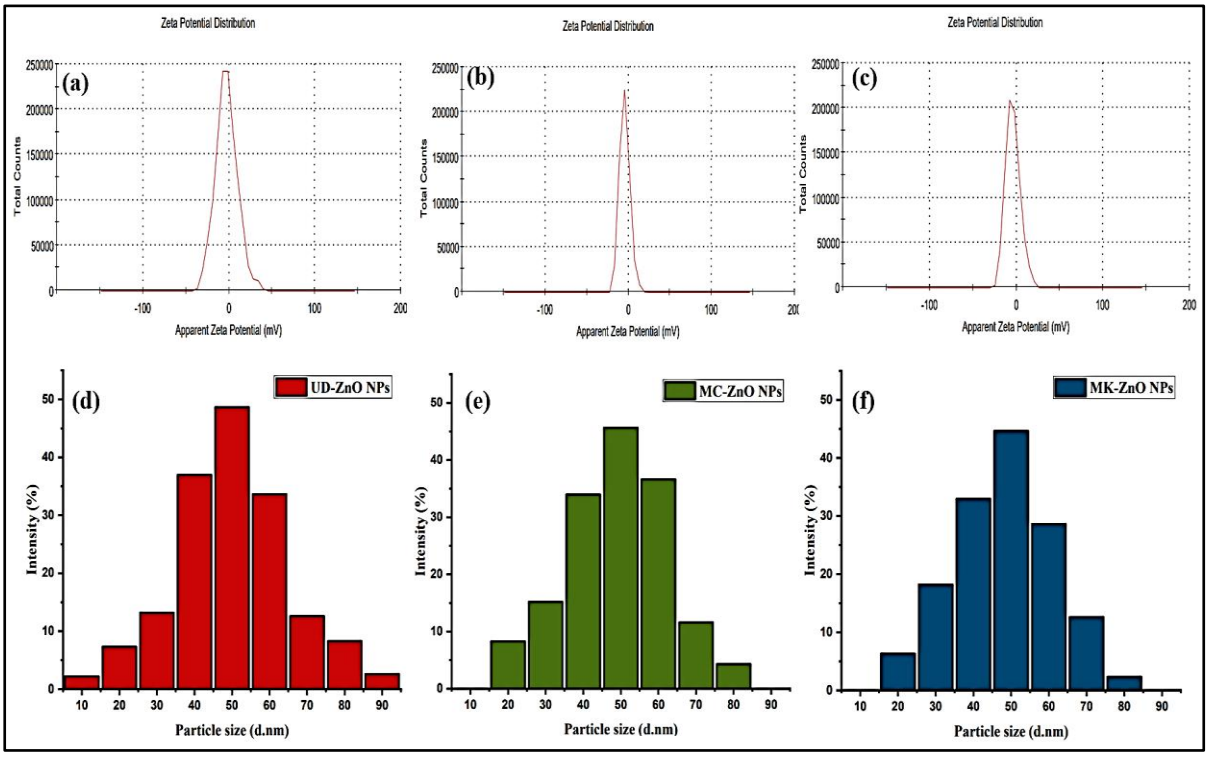


Figure 6.12: Zeta potential and dynamic light scattering (DLS) of *U. dioica* (UD), *M. chamomilla* (MC), *and M. koenigii* (MK) ZnO nanoparticles.

The distribution curves of UD-ZnO, MC-ZnO, and MK-ZnO NPs depicted **in Figure 6.12 (d,e,f)**. It displayed a range of particle sizes, with an average particle size of 43.31 nm and sizes ranging from 10.63 nm to 90.37 nm. Moreover, there was a 45.18 nm gap between the nanoparticles' highest and smallest sizes, indicating that the ZnO-NPs were distributed in a restricted range. Additionally, the stability of the nanoparticles was assessed using the zeta potential value (**Figure 6.12-a,b,c**), which was found to be -19.2, -17.4, and -18.5 mV of UD-ZnO, MC-ZnO, and MK-ZnO NPs, respectively, which indicates an excellent stability.

The negative value demonstrated the stability of the nanoparticles and prevented their aggregation. The capping effect of the biomolecules found in the aqueous fractions of *U. dioica, M. chamomilla,* and *M. koenigii* could be the cause of the negative potential value.

6.11.6 Energy Dispersive X-ray Spectroscopy

EDX is an effective technique for determining the elements composition of UD-ZnO, MC-ZnO, and MK-ZnO NPs. The unique atomic structures of each element provide recognizable peaks on the X-ray spectrum.

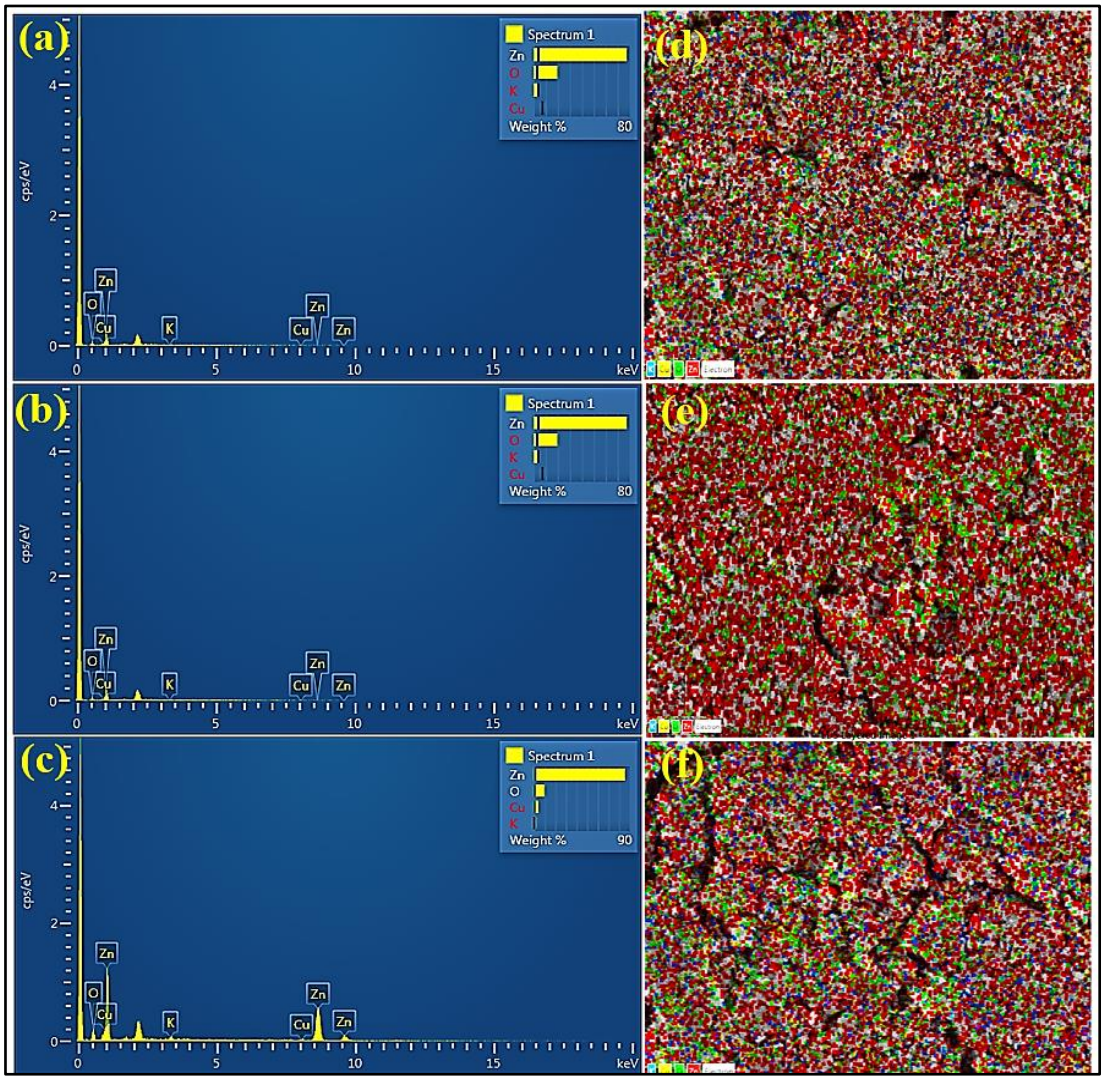


Figure 6.13: EDX (a,b, and c) and molecular mapping (d, e, and f) images of green synthesized ZnO nanoparticles of *U. dioica* (a and d), *M. chamomilla* (b and e), and *M. koenigii* (c and f) (in molecular mapping red=zinc, green=oxygen, yellow=copper, and blue=potassium).

Other elements on the EDX, such as oxygen and carbon, come from the chemical component of the plant extract that is utilized as a reducing agent. The purity and composition of the green engineered ZnO-NPs are displayed in the EDX spectrum **(Figure 6.13).** Zn emits a strong signal in the EDX spectrum, while Cu, O, and K elements emit weak signals. These weak signals are the result of macromolecules such as enzymes, protein, and carbohydrates that are found in the cell wall of the plant fraction emitting X-rays.

6.12 The anti-bacterial potential of green-engineered ZnO-NPs against S. aureus and E. coli

In the present study, the antimicrobial effect of green engineered ZnO-NPs was examined against Gram negative (*Escherichia coli*) and Gram positive (*Staphylococcus aureus*) bacterial strains following subculturing on Muller-Hinton agar medium.

Figure 6.14: Antibacterial activity of plant extracts (UD, MC, and MK) and their bio synthesized zinc oxide nanoparticles (UD-ZnO, MC-ZnO, and MK-ZnO NPs) against *Escherichia coli* (a,b,c,d,e,f) and *Staphylococcus aureus* (g,h,i,j,k,l). Note: +C = positive control (chloramphenicol), – C=negative control (deionized water), UD = *Urtica dioica* extract, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla* extract, MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK = *Murraya koenigii* extract, MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NPs.

The obtained zone of inhibition of plant extracts (UD, MC, and MK) and their phyto synthesized NPs (UD-ZnO, MC-ZnO, and MK-ZnO NPs) were compared with drug viz., chloramphenicol. The maximum inhibition zone of biosynthesized ZnO-NPs in case of *E. coli* were observed in MK-ZnO NPs (1.5 cm) followed by UD-ZnO NPs (1.2 cm), and MC-ZnO NPs (1.0 cm). Plant extracts also showed great antibacterial activity viz. UD extract (0.9 cm), MC extract (0.5 cm), and MK extract (0.9 cm) as illustrated in **Figure 6.14 (a,b,c,d,e,f).**

The pattern was similar in case of *S. aureus*, highest zone of inhibition was observed in MK-ZnO NPs (1.7 cm) followed by UD-ZnO NPs (1.4 cm), and MC-ZnO NPs (1.4 cm) as shown in **Figure 6.14 (g,h,i,j,k,l).** Plant extracts also showed great antibacterial activity viz. UD extract (0.9 cm), MC extract (0.5 cm), and MK extract (1.0 cm) Due to the existence of peptidoglycan layers, Gram positive strains have more convergent ZnO-NPs penetration into cell membrane than their negative equivalents.

The results suggests that the bacteria are very susceptible to ZnO nanoparticles, which have excellent activity against *E. coli* and *S. aureus*. The plant fractions are rich in amino acids, vitamins, phenols, reducing sugars, and flavonoids, which are essential for binding and capturing ZnO ions. The antibacterial activity measured immediately following synthesis and after storage was found to be equally strong, demonstrating that the activity does not decrease with passing time and confirming the stability of UD-ZnO, MC-ZnO, and MK-ZnO NPs throughout storage processes.

Green-engineered ZnO-NPs have shown wide antibacterial efficacy against Gram-positive and Gram-negative bacteria. Multiple processes are thought to be responsible for ZnO-NPs' antibacterial action. To begin, the NPs tiny size and large surface area allow for enhanced contact and interaction with bacterial cells, resulting in cell membrane breakdown. This can result in cellular content leakage and, eventually, cell death. Furthermore, when exposed to light or in the presence of moisture, ZnO-NPs can produce reactive oxygen species, which can cause oxidative stress and damage to bacterial cells. The release of zinc ions from ZnO-NPs

may potentially contribute to their antibacterial properties. Although the mechanisms for delivering NPs into microbial cells have been identified, more research is still needed to determine exactly how nanoparticles interact with membrane receptors and efflux pumps to start antimicrobial activity. There are four ways that NPs can enter a cell, including adhering to the membrane, damaging microbial DNA by producing ROS from Zn ions, interfering with ATP synthesis and DNA replication from ionic forms of nanoparticles, and eventually, forming thiols or phosphates from interactions with nucleic acid and amino acids moieties. However, the primary mechanism by which zinc oxide interacts with bacteria is through interference with both capsule biosynthesis and central carbon metabolism. Higher concentrations of zinc ions in nanoforms impair virulence by lowering the production of hyaluronic acid capsules, which in turn inhibits several crucial enzymes that catabolize glucose and changes the expression of carbon catabolic pathways.

The green fabricated ZnO-NPs can be used for both diagnostic and therapeutic purposes for a variety of pathological diseases because to their promising properties. The destructive activity of UD-ZnO, MC-ZnO, and MK-ZnO NPs at minimum inhibitory levels for bacteria is most likely caused by its ability to damage cell membrane structures and permeability barriers with loss of chemiosmotic control. The particular methods of antibacterial activity can differ based on the size, surface features, and concentration of ZnO-NPs, as well as the characteristics of the bacterial strains being targeted. The combination of intracellular ROS generation, membrane damage, zinc ion release, and interaction with biomolecules all contribute to the antibacterial activity of green-engineered ZnO-NPs.

6.13 Dose-dependent Antioxidant Potential of biosynthesized ZnO-NPs using DPPH and ABTS

DPPH and ABTS are two common chemical compounds used as reagents to test the antioxidant activity of substances such as natural extracts. Because of their capacity to behave as stable free radicals and their simple colorimetric detection techniques, these chemicals are commonly used in antioxidant assessments.

The antioxidant and antiradical capacity of green engineered UD-ZnO, MC-ZnO, and MK-ZnO NPs and their plant extracts is frequently assessed using the DPPH method. Antioxidants are considered to have the ability to donate hydrogen, which helps them scavenge DPPH radicals. When electron/hydrogen donor molecules interact with the organic nitrogen radical,

a yellow or brownish radical solution with reduced absorbance is produced. The maximum UV-Vis spectral range of DPPH is 515-520 nm. The antioxidant capacity was determined at three different concentrations (0.1, 0.2, and 0.3 mg/ml). In this study, MK-ZnO NPs (72.3-92.5%) exhibited a greater capacity to scavenge DPPH free radicals than the UD-ZnO (68.4-81.9%) and MC-ZnO (54.1-72.7%) NPs, as shown in **Figure 6.15 (a)**. ZnO-NPs from all three plants showed the highest scavenging efficiency when compared to their aqueous extracts. The redox potential of phenolic components, which serve as reducing agents, is the main determinant of the antioxidant activity of biosynthesized ZnO-NPs.

The antioxidant properties of the hydrophilic and hydrophobic bioactive compounds found in the green fabricated ZnO-NPs are investigated using the unstable free radical ABTS. The maximum UV-Vis spectral range of ABTS is 415 nm. The antioxidant capacity was determined at three different concentrations (0.1, 0.2, and 0.3 mg/ml). These results suggest that biosynthesized MK-ZnO NPs (71.9-93.4%) have stronger antioxidant and antiradical properties, and are more effective than UD-ZnO (64.6-84.5%) and MC-ZnO (52.7-78.9%) at scavenging ABTS free radicals. In the samples obtained, it was observed that biosynthesized ZnO-NPs had the maximum radical-scavenging capacity of ABTS, followed by their aqueous extracts, as shown in **Figure 6.15 (b)**. The finding suggests that the DPPH and ABTS radical scavenging activities were dose-dependent as the concentration of biosynthesized ZnO-NPs increased, scavenging activities against both radicals were also increased

The antioxidant abilities of ZnO-NPs synthesized from UD, MC, and MK extracts was evaluated and compared to the ABTS and DPPH assays. The antioxidant capacity was determined at three different concentrations (0.1, 0.2, and 0.3 mg/ml). The finding suggests that the DPPH and ABTS radical scavenging activities were dose-dependent as the concentration of biosynthesized ZnO-NPs increased, scavenging activities against both radicals were also increased (the antioxidant potential for ABTS and DPPH was 63.3-83.5% and 56.7-77.1%, respectively).

ZnO nanoparticles from all three plants (UD, MC, and MK) demonstrated the highest scavenging efficiency when compared to their aqueous extracts. The findings are contradictory since some studies found higher antioxidant activity in ZnO-NPs while others found the reverse. Given the huge number of research reporting both types of results, both outcomes seem possible.

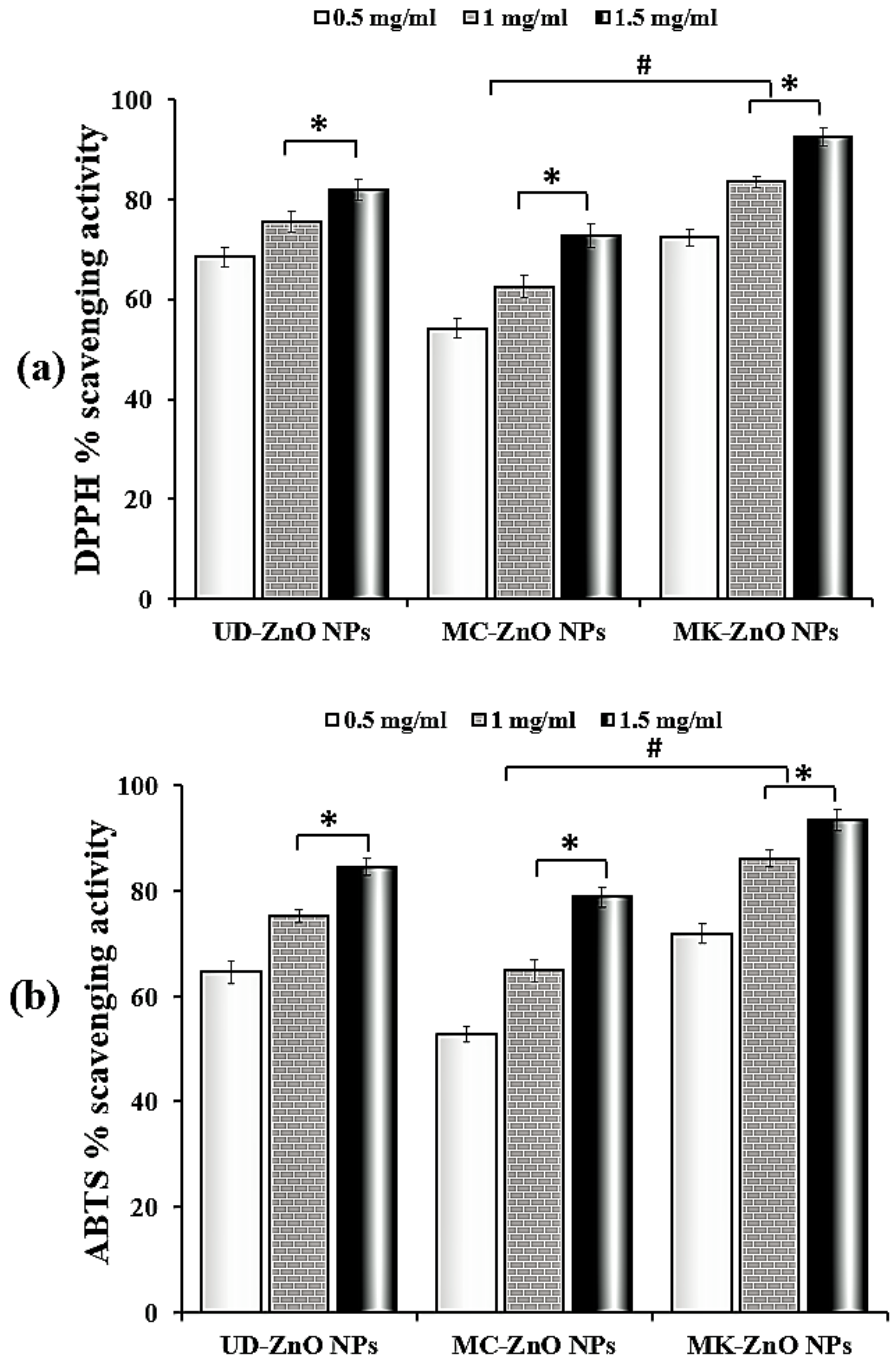


Figure 6.15: The effect of different concentrations (0.1, 0.2, and 0.3 mg/ml) of *Urtica dioica* (UD), *Matricaria chamomilla* (MC), *Murraya koenigii* (MK) synthesized ZnO nanoparticles (UD-ZnO, MC-ZnO, and MK-ZnO NPs) in the DPPH (a) and ABTS (b) free radical scavenging test. Data are shown as the mean±SD for n = 3. Statistically significance is ascribed as **P*<0.05 (intra group) and #*P*<0.05 (inter group) compared with 0.5 mg/ml of the respective groups. UD = *Urtica dioica* extract, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla* extract, MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK = *Murraya koenigii* extract, MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NPs.

6.14 *Trypan blue staining in tissues of green engineered ZnO-NPs exposed D. melanogaster*

Trypan blue staining is a method extensively used in biomedical research for evaluating cell viability and tissue morphology. It entails the use of Trypan blue, a crucial dye that selectively colours dead or injured cells while leaving live cells unstained. The trypan blue staining was carried out in the tissues and whole larvae of *Drosophila* to see if exposure to rotenone generates any tissue damage **(Figure 6.16)**.

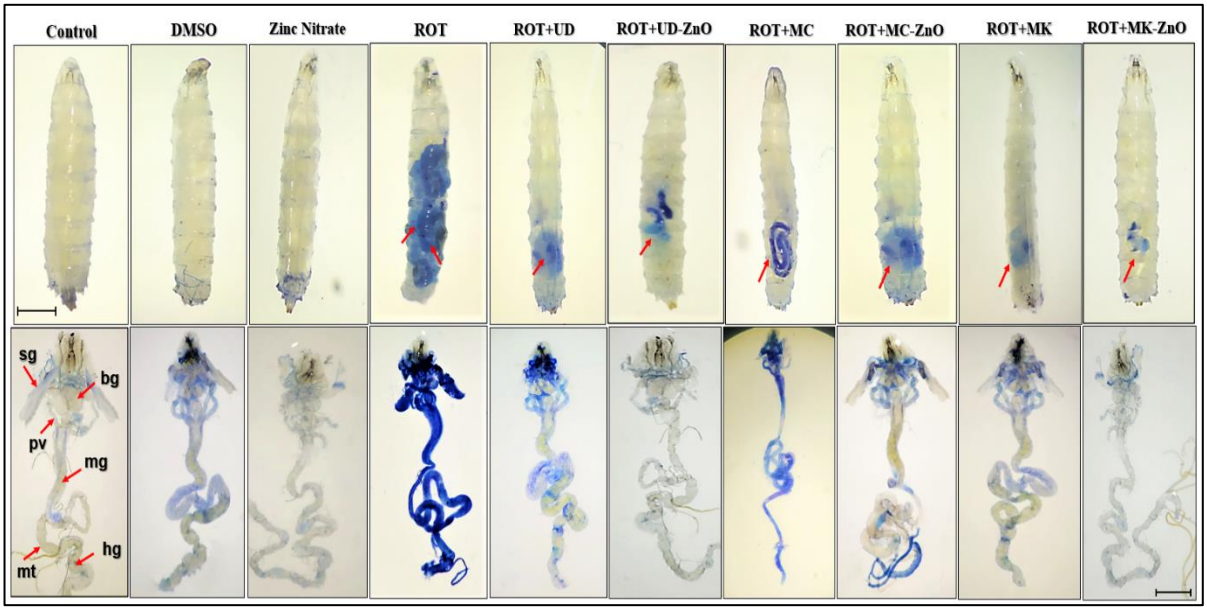


Figure 6.16. Dye exclusion test using trypan blue staining in the third instar larvae exposed to rotenone and cotreated with UD-ZnO, MC-ZnO, and MK-ZnO, as illustrated in the upper panel. The lower panel shows dissected third instar larvae dyed with trypan blue. ROT 500 µM was administered to 72-hour (±2 h) old larvae (early third instar) of *Drosophila melanogaster* (Oregon R+) alone or in association with UD-ZnO, MC-ZnO, and MK-ZnO, respectively for 48 hours. The upper panel's arrows demonstrate cytotoxicities in the whole larvae using trypan blue staining. Note: sg= salivary glands, pv= proventriculus, bg= brain ganglia, mt= malpighian tubules, mg= midgut, and hg = hind gut. The bar represents 100 µm. ROT= rotenone; UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla* synthesized Zinc Oxide NPs and MK = *Murraya koenigii* synthesized Zinc Oxide NPs.

In 96% of the larvae treated to ROT, the whole larva and its tissues exhibited blue staining (midgut, brain ganglia, gastric caeca, salivary gland, and malpighian tubules). ROT co-exposed with MK-ZnO NPs demonstrated considerably lesser blue staining in the tissues mentioned above and the whole larvae when compared to the ROT plus UD-ZnO and ROT plus MC-ZnO NPs groups, respectively.

The trypan blue staining principle is based on the differential permeability of the cell membrane in living and dead cells. Trypan blue is a water-soluble dye that cannot penetrate living cells' undamaged cell membranes. However, the cell membrane loses its integrity in dead or injured cells, enabling trypan blue to enter the cell and stain its contents. Plant biomolecules significantly enhanced cell viability. Biogenic ZnO nanoparticles showed protective properties due to the activation of antioxidant defence mechanisms or the presence of bioactive molecules that quench free radicals.

6.15 *Biosynthesized ZnO-NPs enhanced AChE activity in Rotenone exposed D. melanogaster*

It was observed in this study that the third instar larvae for 24 hours exposed to ROT showed a significant statistically (P<0.001) decrease in acetylcholinesterase activity when compared to either DMSO or control, with 68.91% lower AChE levels in the said group. These higher levels of acetylcholinesterase were clearly considerable when compared to ROT alone treated groups, with just 20.65% inhibition being seen as opposed to control when ROT and MK-ZnO NPs were co-exposed. Interestingly, there was a considerable improvement in the AChE levels in the ROT plus UD-ZnO and ROT plus MC-ZnO NPs groups (31.85% and 43.75% inhibition, respectively). Plant fractions also demonstrated encouraging effects in the suppression of rotenone-induced acetylcholinesterase inhibition (UD-44.53%, MC-52.03%, and MK-33.79% inhibition). After 48 hours, ROT alone exposed organisms had the highest amount of AChE inhibition (77.92%) opposed to control/untreated larvae, while the ROT plus MK-ZnO NPs group had the highest degree of rescue from ROT-induced neurotoxicity (10.42%). Additionally, the ROT plus UD-ZnO and ROT plus MC-ZnO groups considerably had greater AChE levels than the ROT-treated group (22.64% and 35.35% inhibition, respectively) as illustrated in the **Figure 6.17-a.** Plant extracts also showed promising results against rotenone induced acetylcholinesterase inhibition (UD-34.16%, MC-44.46%, and MK-27.64%). According to obtained results, it suggests that the biosynthesized ZnO nanoparticles effectively enhanced the activity of AChE in *Drosophila melanogaster* that were exposed to rotenone. This implies that the Phytosynthesized ZnO-NPs may have a protective effect on the nervous system by counteracting the inhibitory effects of rotenone on AChE activity. Both plant-synthesized ZnO nanoparticles and plant extracts have the potential to rescue AChE inhibition, the effectiveness of each approach can vary depending on the specific application, the plant species involved, and the characteristics of the nanoparticles or extracts used.

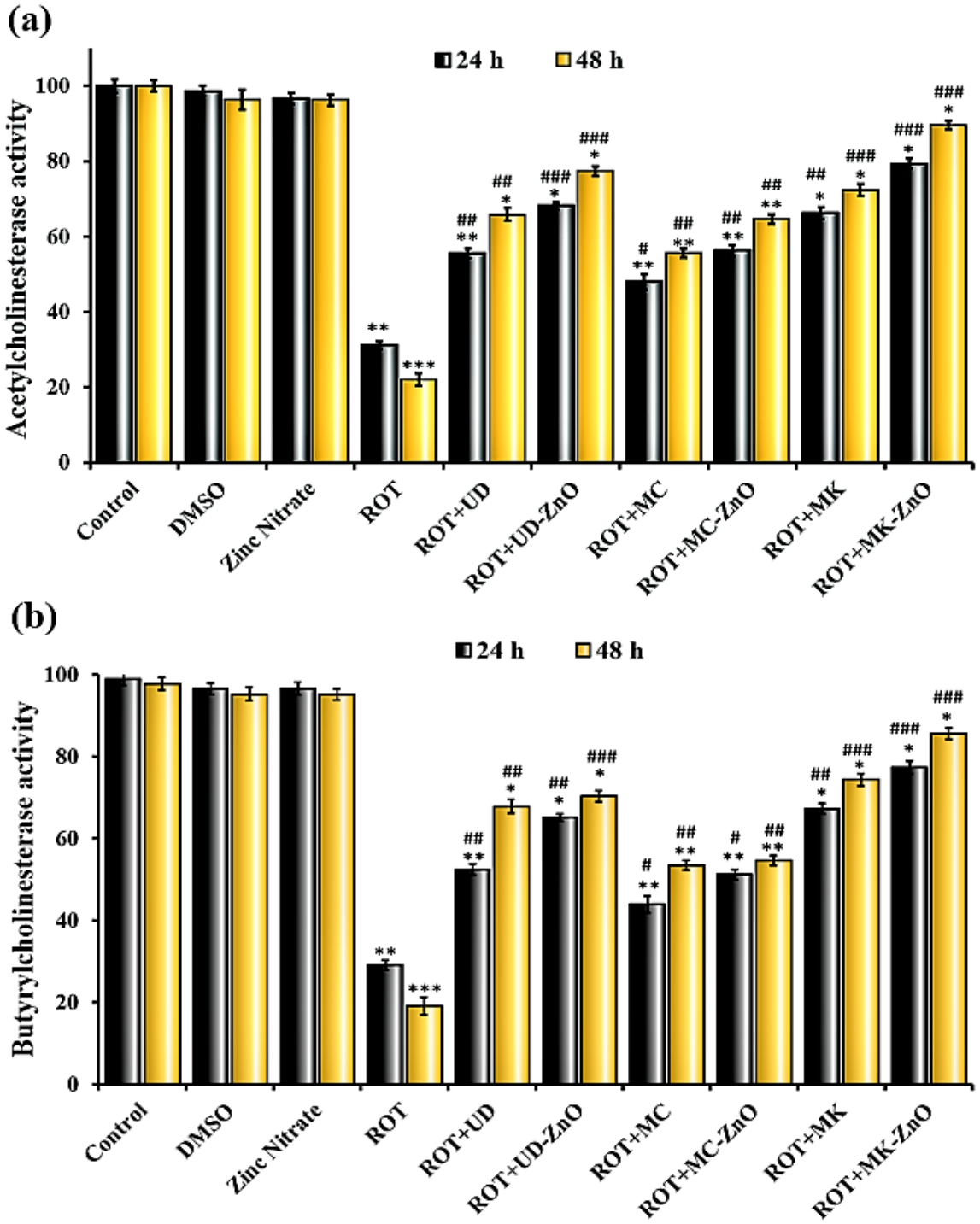


Figure 6.17: Acetylcholinesterase (a) and butrylcholinesterase (b) activities in the third instar larvae of *D. melanogaster* (Oregon R^{+}) exposed to 500 µM ROT alone or in combination with UD, MC, and MK extracts and their synthesized nanoparticles (ZnO-NPs) for 24 and 48 h. Data represent mean ± SD (n = 3); significance ascribed as ** $p < 0.01$, *** $p < 0.001$ vs. control or DMSO control. $^{\#}$ is ascribed as significance at $p < 0.05$, $^{\#\#\#}$ $p < 0.001$ as compared with 500 µM rotenone. Note: UD = *Urtica dioica*, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla extract,* MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK= *Murraya koenigii extract,* MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NPs.

6.16 Ameliorative effects of ZnO-NPs on rotenone induced BChE inhibition in D. melanogaster

In this study, third instar larvae of Oregon R^+ exposed to ROT for 24 hours revealed a statistically significant (P<0.001) reduction in butyrylcholinesterase activity as compared to either DMSO or control, with 66.31% lower BChE levels in the aforementioned group. When ROT and MK-ZnO NPs were co-exposed, these greater levels of butyrylcholinesterase were obviously significant when compared to ROT alone treated groups, with just 24.65% inhibition compared to control. Surprisingly, BChE levels improved significantly in the ROT plus UD-ZnO and ROT plus MC-ZnO NPs groups (35.6% and 45.7% inhibition, respectively). Plant fractions also demonstrated encouraging effects in the suppression of rotenone-induced butyrylcholinesterase inhibition (UD-44.53 %, MC-52.03 %, and MK-33.79 inhibition).

After 48 hours, ROT-exposed organisms had the highest level of BChE inhibition (75.12%) compared to control/untreated larvae, whereas ROT + MK-ZnO NPs had the highest level of rescue from ROT-induced neurotoxicity (13.42%). Furthermore, as shown in **Figure 6.17-b**, the ROT plus UD-ZnO and ROT plus MC-ZnO groups exhibited significantly higher BChE levels than the ROT-treated group (27.63% and 36.35% inhibition, respectively). Plant extracts also showed promising results against rotenone induced butyrylcholinesterase inhibition (UD-34.16 %, MC-44.46 %, and MK-27.64).

According to the findings, biosynthesized ZnO nanoparticles efficiently increased the activity of BChE in *Drosophila melanogaster* exposed to rotenone. This suggests that the nanoparticles may protect the nervous system by counteracting rotenone's inhibitory effects on BChE activity. Although both plant-derived ZnO nanoparticles and plant extracts have the ability to restore BChE inhibition, the efficacy of each strategy varies depending on the specific application, plant species involved, and the properties of the nanoparticles or extracts employed.

6.17 *Modulatory effects of green ZnO-NPs on rotenone induced catalase (CAT) alterations*

Rotenone, as mentioned earlier, is a mitochondrial complex I inhibitor that induces oxidative stress and disrupts the antioxidant defense system. This can lead to an increase in ROS levels and an increase in CAT activity, which is responsible for breaking down hydrogen peroxide into water and oxygen. To investigate whether green ZnO-NPs could mitigate the rotenone-induced alterations in CAT assay were carried out in *Drosophila*.

The catalase activity in the tissues of third instar larvae of *Drosophila* exposed to ROT (500 μM) increased statistically significantly (P<0.001). When compared to the control group, the catalase activity of the larvae in the ROT group was increased after 24 hours. The lowest CAT activity was observed in ROT co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs, and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) also shown promising effects in suppressing rotenone-induced CAT activity. Many plants contain bioactive compounds such as polyphenols, flavonoids, and other antioxidants that have the potential to scavenge reactive oxygen species (ROS) and modulate enzymatic activity. After 48 hours, the ROT exposure has significant increase in CAT activity in the larvae compared to the control group. ROT co-exposed with MK-ZnO NPs showed a decrease in CAT activity, followed by ROT+UD-ZnO, and ROT+MC-ZnO NPs **(Figure 6.18-a)**. UD, MC, and MK extracts have been found to regulate the expression of genes involved in antioxidant defense, including CAT. This modulation of gene expression can help to normalize CAT activity in the presence of rotenone. The obtained data were analyzed statistically to determine the effects of rotenone and/or biosynthesized ZnO-NPs on CAT activity in *Drosophila*. Based on the findings, we conclude that green ZnO-NPs protect against rotenone-induced oxidative stress by restoring or strengthening the activity of antioxidant enzymes.

6.18 Ameliorative effects of green ZnO-NPs on rotenone induced superoxide dismutase (SOD) alterations

The effects of green ZnO-NPs on rotenone-induced alterations to antioxidant enzymes, specifically SOD, in *D. melanogaster* have been studied. In this study, it was observed that SOD level increased considerably (P<0.001) in the tissues of third instar *Drosophila* larvae exposed to ROT (500 μM). After 24 hours, the SOD level of the larvae in the ROT group had risen than in the control group. ROT co-exposed with MK-ZnO NPs had the lowest SOD activity, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) have also been demonstrated to decrease rotenone-induced SOD activity. After 48 hours, when compared to the control group, ROT exposure resulted in a substantial increase in SOD level in the larvae. ROT co-exposed with MK-ZnO NPs reduced SOD expression, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs **(Figure 6.18-b)**. UD, MC, and MK extracts also have been reported to affect the expression of antioxidant defence genes such as SOD. Based on the findings, we conclude that green ZnO-NPs protect against rotenone-induced oxidative stress by restoring or strengthening the activity of antioxidant enzymes.

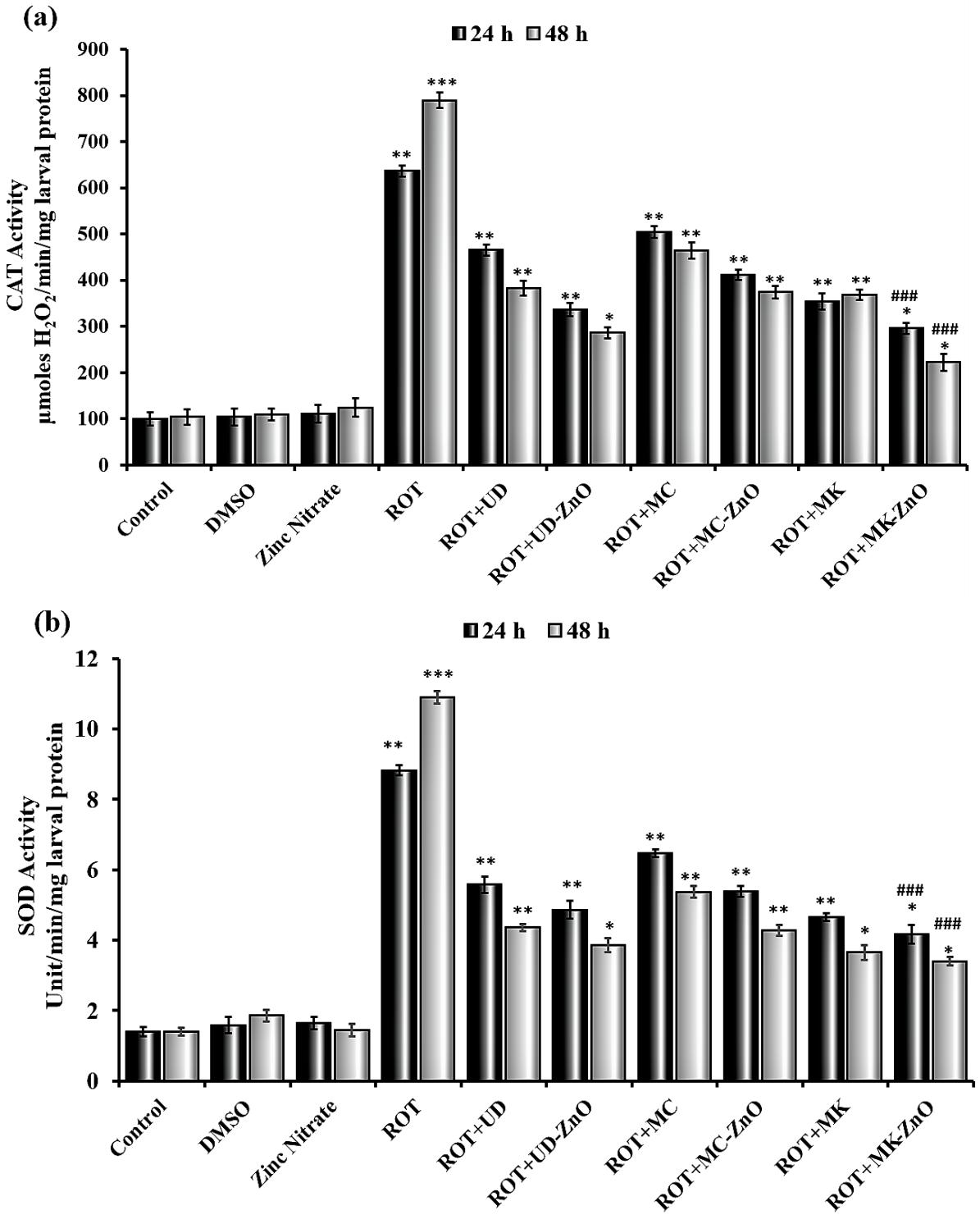


Figure 6.18: CAT (a) and SOD (b) activities in the third instar larvae of *D. melanogaster* (Oregon R^+) exposed to 500 µM ROT alone or in combination with UD, MC, and MK extracts and their synthesized nanoparticles (ZnO-NPs) for 24 and 48 h. Data represent mean ± SD (n = 3); *p<0.05, ** p< 0.01, *** p< 0.001 vs. untreated/control or DMSO control. ## ascribed as significance at p< 0.01, ### p< 0.001 compared with 500 µM rotenone. Note: UD = *Urtica dioica*, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla extract,* MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK= *Murraya koenigii extract,* MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NPs.

6.19 Lipid peroxidation (MDA content)

In this research, MDA level was significantly higher (P<0.001) in the tissues of third instar *Drosophila* larvae exposed to ROT (500 µM). After 24 hours, the MDA level in the ROT group was higher than in the control group. The lowest MDA concentration was found in ROT co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) reduced rotenone-induced MDA level as well. After 48 hours, when compared to the control group, ROT exposure resulted in a significant rise in MDA levels in the larvae. MDA expression was decreased when ROT was co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs **(Figure 6.19-a**). UD, MC, and MK extracts all showed improved effects on the reduction of lipid peroxidation induced by rotenone. Rotenone exposure led to increased oxidative stress which further led to higher levels of LPO in cells. Plant-derived ZnO-NPs demonstrated the enhanced protective effect against lipid peroxidation. ZnO-NPs possess intrinsic antioxidant properties and can scavenge ROS. By reducing oxidative stress, these nanoparticles can help mitigate the generation of lipid peroxides, thereby lowering LPO levels.

6.20 Protein carbonyl content (PC content)

To investigate the protective effects of phyto-synthesized ZnO-NPs on rotenone-induced increased protein carbonyl content study was carried out. In this work, PC content was significantly increased (P<0.001) in the tissues of third instar larvae of *D. melanogaster* exposed to ROT (500 µM). After 24 hours, the PC concentration in the ROT group was higher than in the control group. The reduced PC concentration was found in ROT co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) reduced rotenone-induced PC level as well. After 48 hours, when compared to the control group, ROT exposure resulted in a significant rise in PC level in the larvae. PC expression was decreased when ROT was co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs **(Figure 6.19-b**). UD, MC, and MK extracts all showed improved effects on the reduction of protein carbonyl induced by rotenone. Protein carbonylation occurs as a result of oxidative damage to proteins, leading to the formation of carbonyl groups on amino acid residues. Green ZnO-NPs, with their intrinsic antioxidant properties, may have the potential to scavenge ROS and reduce oxidative stress. This reduction in oxidative stress could potentially result in the decreased formation of protein carbonyls induced by rotenone.

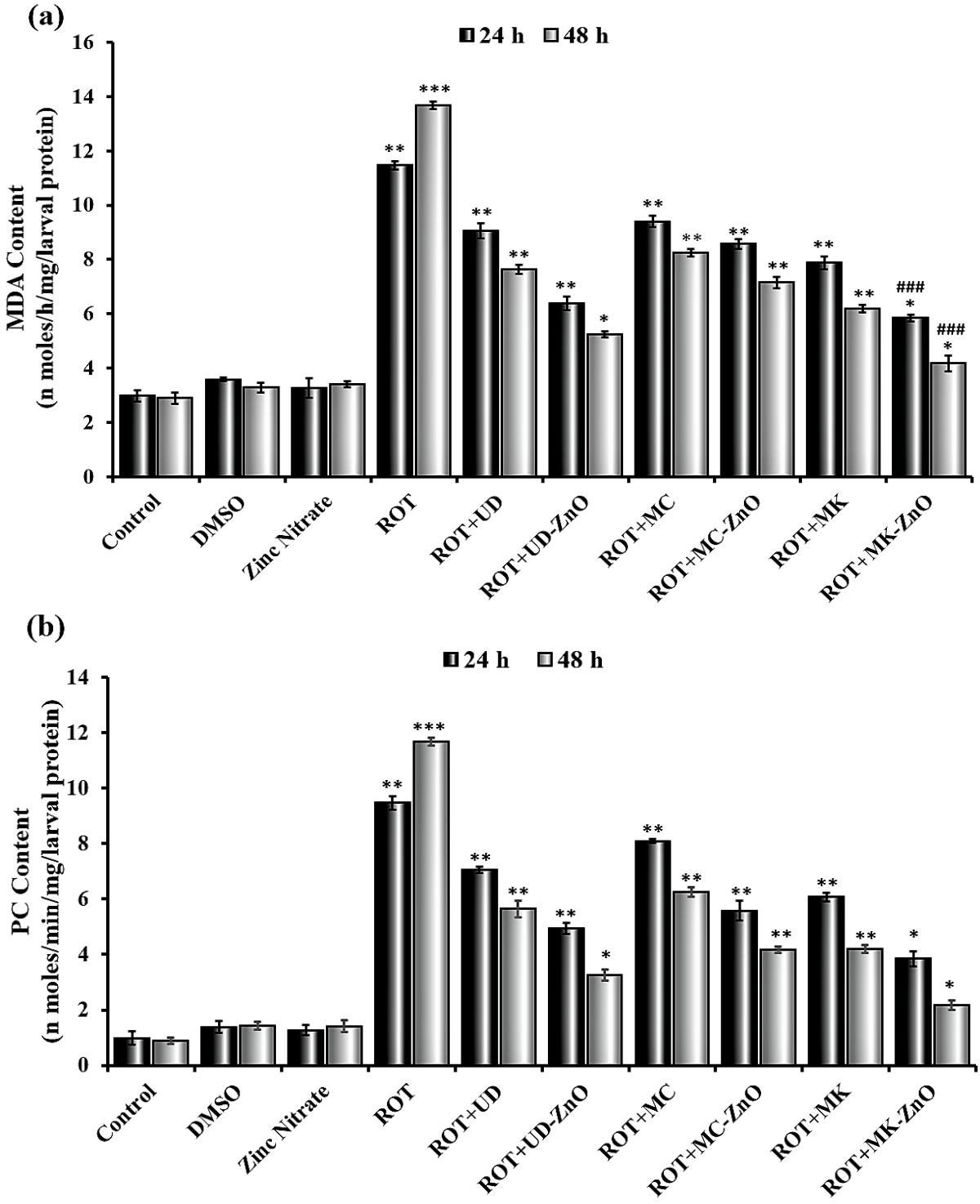


Figure 6.19: LPO (a) and PC (b) content in the third instar larvae of *D. melanogaster* (Oregon R^+) exposed to 500 µM ROT alone or in combination with UD, MC, and MK extracts and their synthesized nanoparticles (ZnO-NPs) for 24 and 48 h. Data represent mean ± SD (n = 3); **p*<0.05, ** *p*< 0.01, *** *p*< 0.001 vs. untreated/control or DMSO control. ## ascribed as significance at *p*< 0.01, ### *p*< 0.001 compared with 500 µM rotenone. Note: UD = *Urtica dioica*, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla extract,* MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK= *Murraya koenigii extract,* MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NPs.

6.21 Decreased protein concentration in Drosophila exposed with ROT after 24 and 48 h

Rotenone can affect dopaminergic neurons in *Drosophila*. Dopamine-related proteins, such as tyrosine hydroxylase (TH) or dopamine transporters, may be altered in response to rotenone exposure, potentially leading to changes in protein concentration. The total protein concentration in the tissues of third instar larvae of *Drosophila* exposed to ROT (500 µM) decreased statistically significantly (P<0.001). When compared to the control group (14.98±0.211 mg/g), the protein concentration of the larvae in the ROT (6.41±0.258 mg/g) group was lower after 24 hours. The highest protein content was observed in ROT co-exposed with MK-ZnO NPs (11.09±0.214 mg/ml), followed by ROT+UD-ZnO NPs (10.08±0.212 mg/g), and the lowest in ROT+MC-ZnO NPs (9.06±0.235 mg/g). Plant extracts such as UD (8.55±0.11 mg/ml), MC (7.09±0.14 mg/ml), and MK (9.27±0.17 mg/ml) also enhanced protein concentration as well. After 48 hours, the ROT (5.08±0.258 mg/g) exposure has reduced the protein content in the larvae compared to the control group (14.77±0.328 mg/g). ROT co-exposed with MK-ZnO NPs showed an increase in protein concentration (12.9±0.323 mg/g), followed by ROT+UD-ZnO (11.85±0.231 mg/g), and ROT+MC-ZnO NPs (10.65±0.256 mg/g) **(Figure 6.20-a)**. Protein concentration was also increased by plant extracts including UD (9.94±0.24 mg/g), MC (8.04±0.24 mg/g), and MK (10.58±0.17 mg/g).

6.22 Reduced glutathione (GSH) level in Drosophila exposed with ROT after 24 and 48 h

The glutathione content in the tissues of third instar larvae of *Drosophila* exposed to ROT (500 µM) decreased statistically significantly (P<0.001). When compared to the control group (1.48%), the GSH concentration of the larvae in the ROT (71.30%) group was reduced after 24 hours. Among the ZnO-NPs synthesized groups, the highest GSH content was observed in ROT co-exposed with MK-ZnO NPs (24.64% reduction), followed by ROT+UD-ZnO NPs (39.84%), and the lowest in ROT+MC-ZnO NPs (45.75%). Plant extracts such as UD (44.52%), MC (59.02%), and MK (37.70%) also enhanced GSH concentration as well.

After 48 hours, the ROT (81.12%) exposure has reduced the GSH content in the larvae compared to the control group (2.75%). ROT co-exposed with MK-ZnO NPs showed an increase in GSH concentration (16.41%), followed by ROT+UD-ZnO (28.63%), and ROT+MC-ZnO NPs(36.34%) **(Figure 6.20-b)**. Glutathione concentration was also increased by plant extracts including UD (38.15%), MC (47.45%), and MK (27.63%).

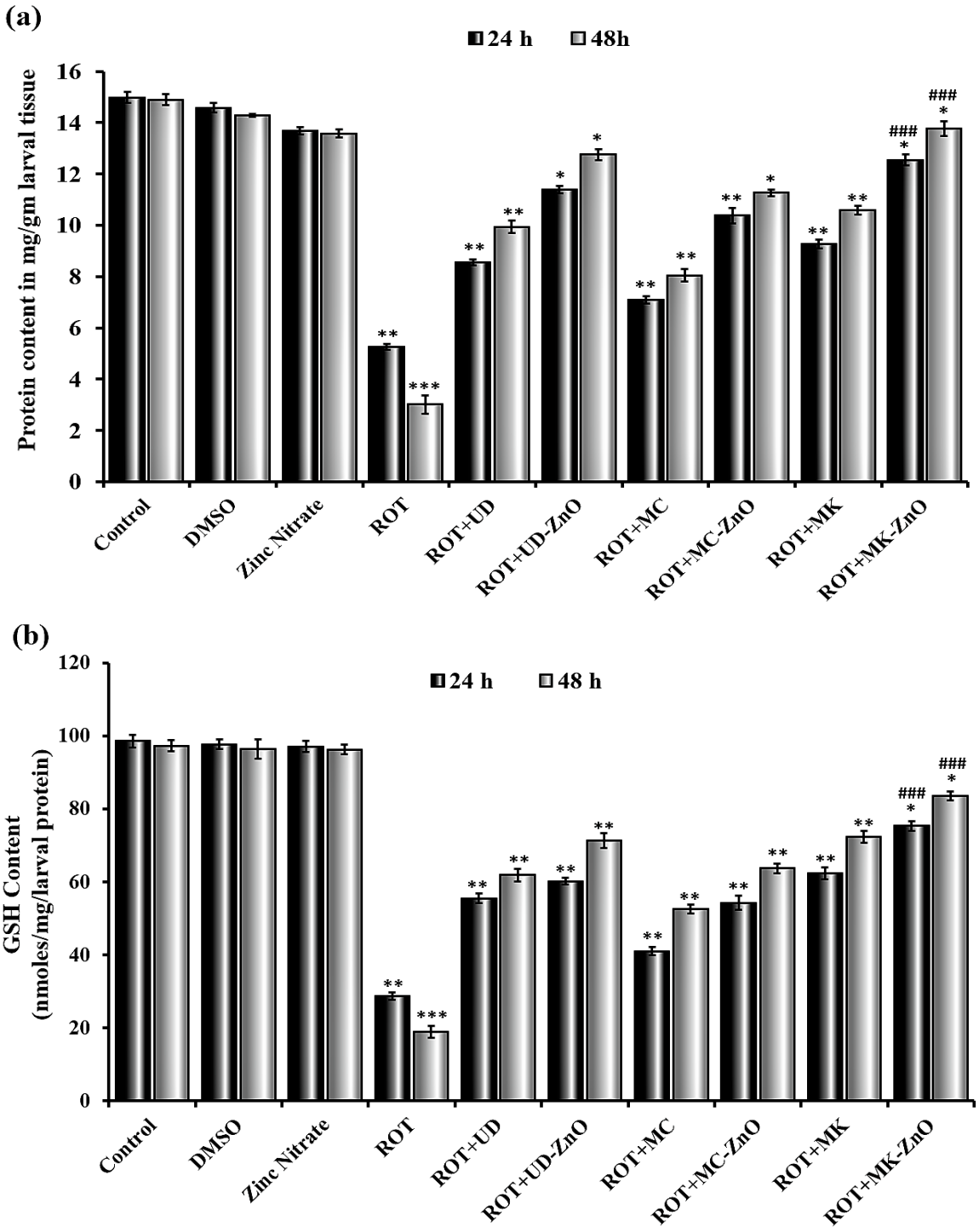


Figure 6.20: Protein content (a) and GSH (b) content in the third instar larvae of *D. melanogaster* (Oregon R^+) exposed to 500 µM ROT alone or in combination with UD, MC, and MK extracts and their synthesized nanoparticles (ZnO-NPs) for 24 and 48 h. Data represent mean ± SD (n = 3); *p<0.05, ** p< 0.01, *** p< 0.001 vs. untreated/control or DMSO control. ## ascribed as significance at p< 0.01, ### p< 0.001 compared with 500 µM rotenone. Note: UD = *Urtica dioica*, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla extract,* MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK= *Murraya koenigii extract,* MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NPs.

6.23 *Significant variation in locomotor activity of ROT treated Drosophila.*

Rotenone-induced mitochondrial dysfunction has also been associated with oxidative stress and the generation of ROS, which can further contribute to the impairment of locomotor activity in *Drosophila*. The highest climbing potential was shown by the control, DMSO, and zinc nitrate-treated flies in 30 seconds (only 8.33, 11.41, and 13.47 % decrease, respectively). The maximum decrease was detected in ROT-treated *Drosophila* (65.5%), and flies had trouble to climb the walls of cylinder. Different groups with ROT and plant-mediated ZnO-NPs demonstrate variable degrees of improvement in their climbing abilities. The ability of flies to ascend was increased by all green ZnO-NPs as illustrated in **Figure 6.21-a**.

Among the synthesized ZnO-NPs groups, ROT+MK-ZnO NPs (17.62%) exhibits the minimum reduction, followed by the ROT+UD-ZnO (27.85%) and ROT+MC-ZnO (38.25%) showed the greatest reduction in climbing activity. Obtained results suggests that plant extracts as well as their synthesized ZnO-NPs enhanced locomotor activity in *Drosophila melanogaster* that had been exposed to rotenone. An unpaired student t-test was used to compare the mean±SEM in order to examine any significant difference. The significance level was determined at $P<0.001$.

6.24 Phyto-synthesized ZnO-NPs enhanced Memory in Rotenone exposed D. melanogaster

Rotenone is known to cause mitochondrial malfunction and oxidative stress, which can impair neuronal function and cognitive processes such as memory. The highest memory ability was shown by the control, DMSO, and zinc nitrate-treated flies (only 5.33, 7.33, and 6.47 % reduction, respectively). The maximum decrease was detected in ROT-treated *Drosophila* (78.6%), and flies was observed random movement at the T-point of the apparatus and the flies were slow enough to reach the T-point. The ability of flies to memorize was increased by all green ZnO-NPs as illustrated in **Figure 6.21-b**.

Among the synthesized ZnO-NPs groups, ROT+MK-ZnO NPs (14.33%) exhibits the minimum reduction, followed by the ROT+UD-ZnO (24.33 %) and ROT+MC-ZnO (31.33%) showed the greatest reduction in memory ability. Obtained results suggests that plant extracts as well as their synthesized ZnO-NPs enhanced memory in *Drosophila melanogaster* that had been exposed to rotenone. An unpaired student t-test was used to compare the mean±SEM in order to examine any significant difference. The significance level was determined at $P<0.001$.

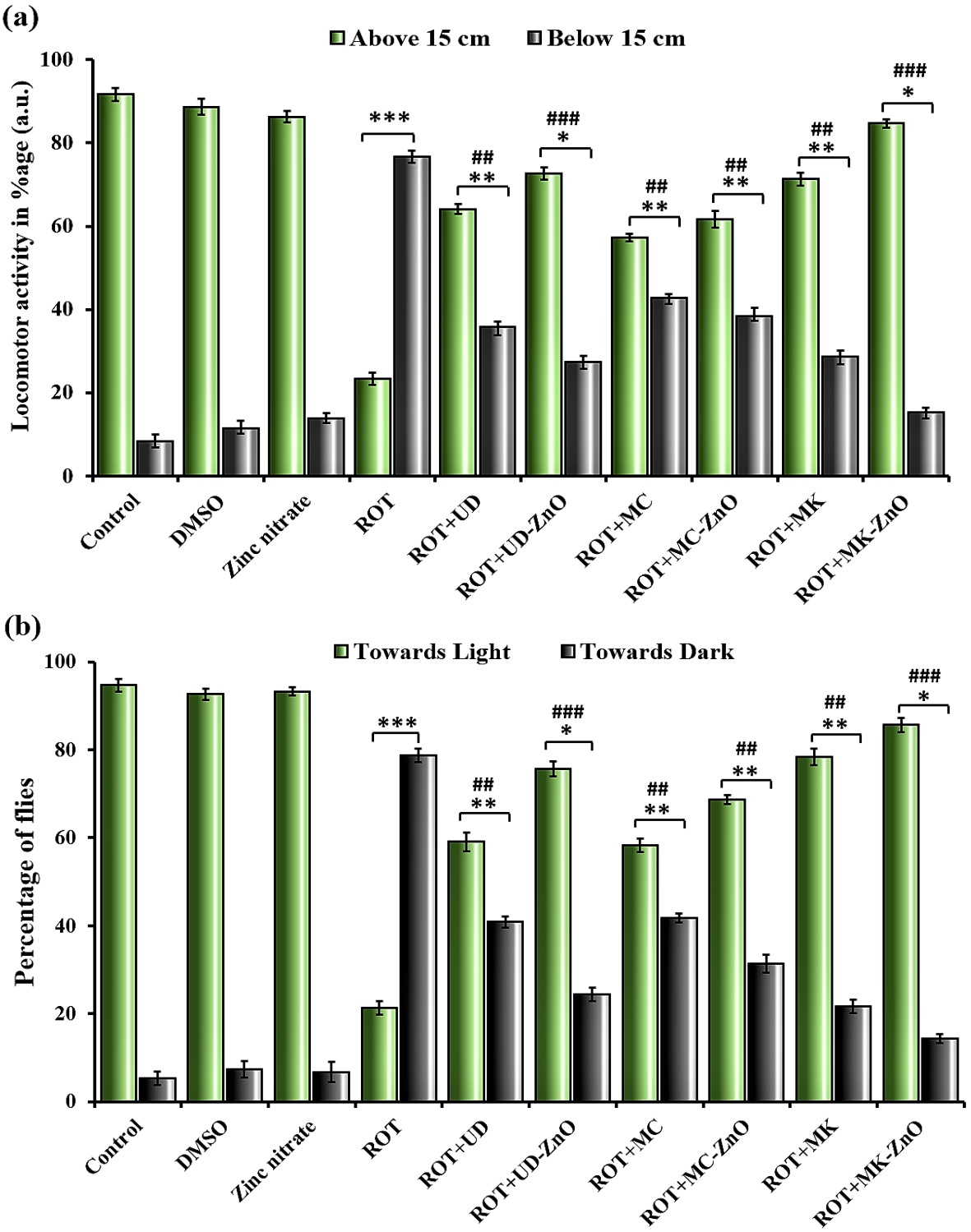


Figure 6.21: Climbing activity (a) and Memory ability (b) of *Drosophila melanogaster* (Oregon R+) flies exposed to ROT (500 µM) alone or in association with UD-ZnO, MC-ZnO, and MK-ZnO for 120 h; significance is ascribed as*p<0.05, ** p< 0.01, *** p< 0.001 vs. untreated/control or DMSO control. ## ascribed as significance at p< 0.01, ### p< 0.001 compared with 500 µM rotenone. Note: UD = *Urtica dioica*, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla extract,* MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK= *Murraya koenigii extract,* MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NPs.

6.25 ROT affects jumping activity in Drosophila

The normal jumping behavior is dependent on the proper functioning of the nervous system and muscle activity, both of which require adequate energy. When compared to control, DMSO, and zinc nitrate (13.67%, 17.02%, and 16.75%), we saw a considerably reduced jumping activity in the *Drosophila* flies treated with ROT (71.4%). Different groups with ROT and green fabricated ZnO-NPs displayed variable degrees of improvement in their jumping abilities. The ability of flies to ascend was increased by all green ZnO-NPs as illustrated in **Figure 6.22-a**. The lowest reduction is shown by the ROT+MK-ZnO (22.67%), followed by the ROT+UD-ZnO (31.67%), and the maximum reduction is shown by the ROT+MC-ZnO (44.04%). When exposed to rotenone, *Drosophila* exhibit a decrease in jumping activity. This decrease can be attributed to the reduced energy levels caused by the inhibition of complex I. The flies may show a decrease in overall motor activity, including their ability to perform coordinated jumping movements. An unpaired student t-test was used to compare the mean±SEM in order to examine any significant difference. The significance level was determined at $P<0.001$.

6.26 Green fabricated ZnO-NPs elevated crawling activity in ROT treated Drosophila larvae

In order to detect any deficits in their locomotor function and consequent behavioural alterations, the crawling pattern of third instar larvae of *Drosophila* was examined. The basic behaviour of *Drosophila* larval crawling enables us to examine the involvement of genes in the specification of neurons in the entire organism as well as the functions of those neurons. The control, DMSO, and zinc nitrate-treated larvae had the highest level of crawling activity in 1 minute (8.89±0.12, 8.66±0.09, and 8.55±0.98 cm/min, respectively). The largest reduction was seen in *Drosophila* treated with ROT (3.05±0.14 cm/min), and flies had difficulty crawling on petri plates. Different ROT and plant-mediated ZnO-NP groups exhibit varying degrees of improvement in their ability to crawl. All green ZnO-NPs improved the ability of larvae to crawl appropriately, as shown in **Figure 6.22-b**. In this study, it was observed that the crawling ability was highly rescued in ROT+MK-ZnO NPs (7.25±0.25 cm/min), followed by ROT+UD-ZnO (6.76±0.10 cm/min), and ROT+MC-ZnO (5.56±0.05 cm/min) among the synthesised ZnO-NPs groups. The mean and SEM were compared using an unpaired student t-test to determine any difference that could be statistically significant. The significance level was determined at $P<0.001$.

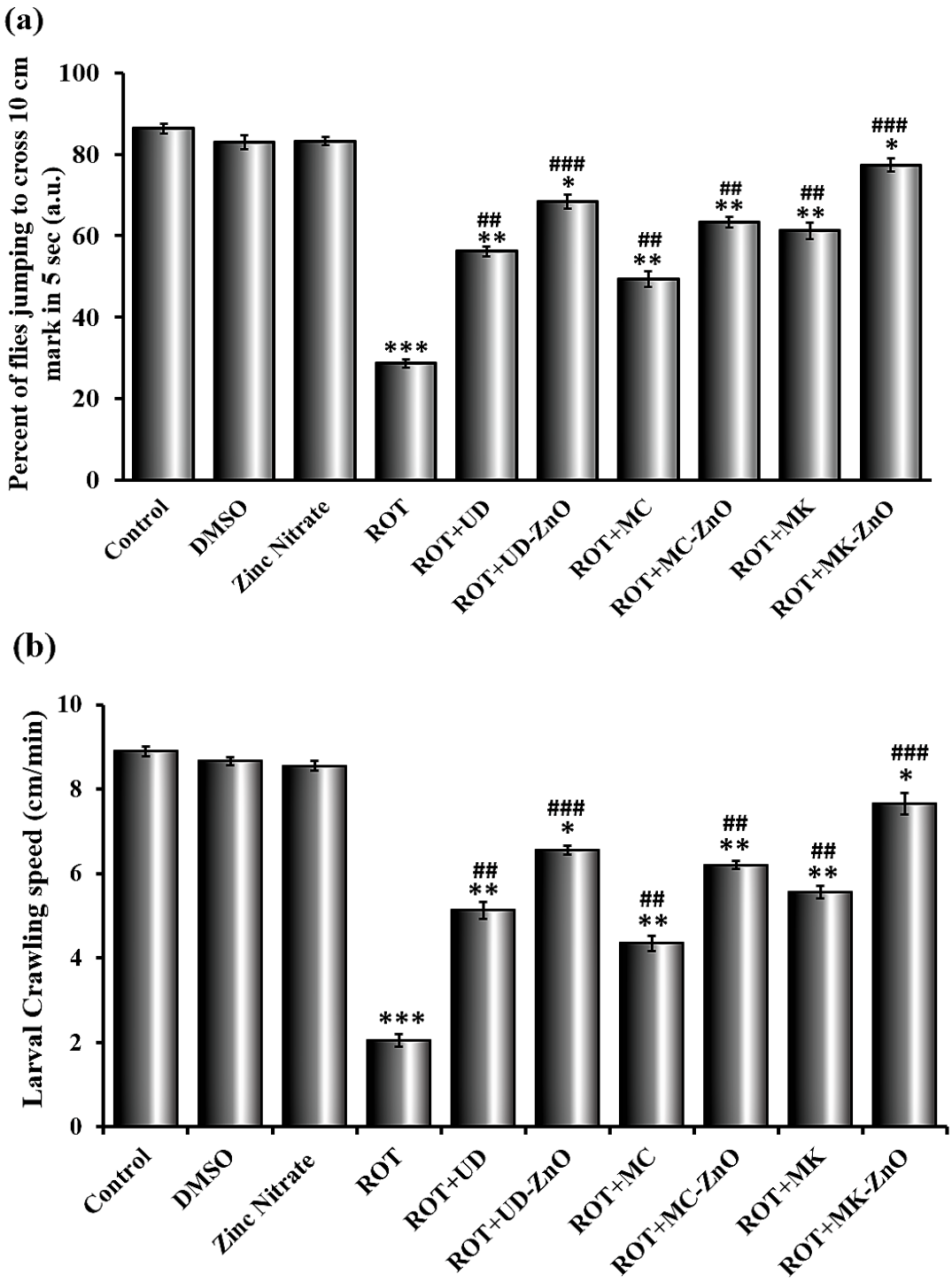


Figure 6.22: Jumping activity (a) and Crawling ability (b) of *Drosophila melanogaster* (Oregon R^{+}) exposed to ROT (500 µM) alone or in association with UD-ZnO, MC-ZnO, and MK-ZnO for 120 h; significance is ascribed as**p*<0.05, ** *p*< 0.01, *** *p*< 0.001 vs. untreated/control or DMSO control. ## ascribed as significance at *p*< 0.01, ### *p*< 0.001 compared with 500 µM rotenone. Note: UD = *Urtica dioica*, UD-ZnO = *Urtica dioica* synthesized Zinc Oxide NPs, MC = *Matricaria chamomilla extract,* MC-ZnO = *Matricaria chamomilla* synthesized Zinc Oxide NPs, MK= *Murraya koenigii extract,* MK-ZnO = *Murraya koenigii* synthesized Zinc Oxide NP.

6.27 Rotenone-induced degeneration in the Drosophila eye

In this study it was observed that the prominent dark area (degeneration) in ROT-treated *Drosophila* compared to control, DMSO, and zinc nitrate-treated flies which showed clear eyes. Different groups with ROT and plant-mediated ZnO-NPs demonstrate variable degrees of improvement as illustrated in **Figure 6.23**. Among the synthesized ZnO-NPs groups, ROT+MK-ZnO exhibits the minimum degeneration, followed by the ROT+UD-ZnO and ROT+MC-ZnO showed the greatest eye degeneration.

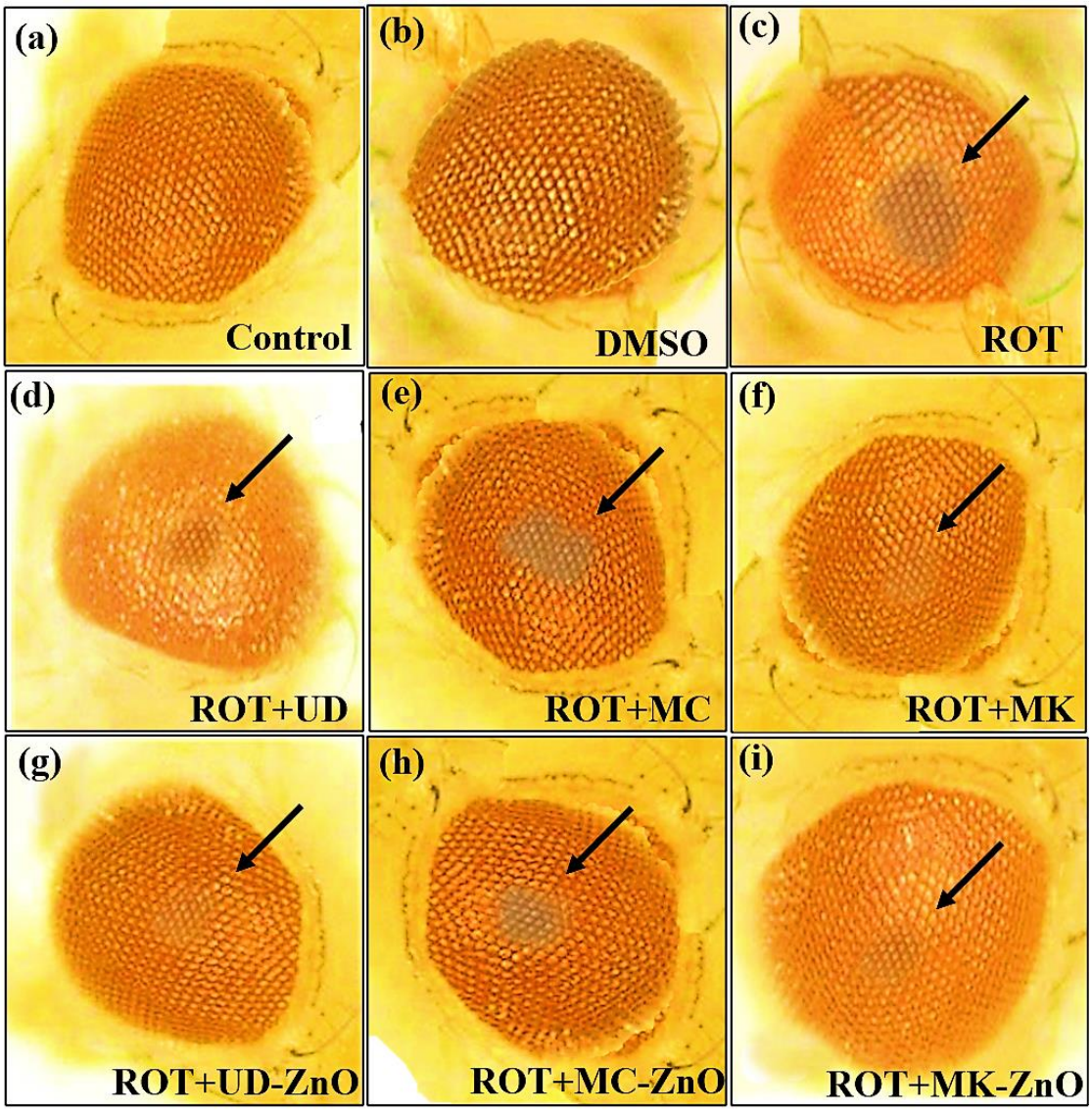


Figure 6.23: Microscopic observations of *Drosophila* eyes exposed to ROT (500 µM) alone or in association with plant extracts (UD, MC, and MK) and their synthesized ZnO-NPs (UD-ZnO, MC-ZnO, and MK-ZnO) for 48 h. The arrows demonstrates degeneration in the eyes of *Drosophila melanogaster*. Note: ROT (Rotenone), UD (*Urtica dioica* extract), UD-ZnO (*Urtica dioica* synthesized Zinc oxide NPs), MC (*Matricaria chamomilla* extract), MC-ZnO (*Matricaria chamomilla* synthesized Zinc oxide NPs), MK (*Murraya koenigii* extract), MK-ZnO = *Murraya koenigii* synthesized Zinc oxide NPs.

6.28 *Rotenone increased tau phosphorylation, amyloid aggregation, and α-synuclein expressions*

To further validate the neuroprotective properties of phyto-synthesized ZnO nanoparticles, we investigated whether there is an up or down regulation by detecting alterations in *APP, tau,* and *α-synuclein* in the third instar larvae of *D. melanogaster* (Oregon R^{+}) exposed to rotenone. Amyloid aggregation refers to the accumulation of misfolded proteins, such as *β-amyloid*, into insoluble clumps or plaques. *Tau* is a protein involved in stabilizing microtubules, which are essential for maintaining the structure and function of nerve cells. In neurodegenerative diseases like AD, tau protein can become abnormally phosphorylated, leading to the formation of tau tangles. *α-synuclein* is a protein that is closely associated with Parkinson's disease and other synucleinopathies.

Real-time Polymerase Chain Reaction (RT-PCR) gene expression studies was performed using *GAPDH* (159 bp) as the housekeeping gene and *APP* (215 bp), *Tau* (105 bp), and *α-Syn* (164 bp) as the target genes as depicted in **Figure 6.24**. In case of *APP* gene, the maximum induction (P<0.05; 425.6% increase) was observed in larvae treated with ROT in comparison to control (98.5%), DMSO (97.6%), and zinc nitrate (98.3%), respectively. Among the synthesized ZnO-NPs groups, ROT+MK-ZnO NPs (99.8%) exhibits the minimum induction, followed by the ROT+UD-ZnO (110.25%) and ROT+MC-ZnO (162.32%) also showed the decrease in gene expression. Plant extracts such as ROT+UD (130.4%), ROT+MC (245.6%), and ROT+MK (105.3%) suppressed the *APP* mRNA expression as well. In terms of *tau* gene, the greatest induction (P<0.05; 429.48% increase) was seen in larvae treated with ROT compared to control (97.5%), DMSO (101.9%), and zinc nitrate (98.5%), respectively. Among the synthesised ZnO-NPs groups, ROT+MK-ZnO NPs (98.5%) demonstrate the least induction, followed by ROT+UD-ZnO (140.6%) and ROT+MC-ZnO (174.6%). Plant extracts such as ROT+UD (246.1%), ROT+MC (325.1%), and ROT+MK (109.3%) also downregulated the *tau* mRNA expression. In case of *α-synuclein*, larvae treated with ROT demonstrated the increase in induction (P<0.05; 444.8% increase) compared to controls (101.5%), DMSO (98.6%), and zinc nitrate (100.5%), respectively. ROT+MK-ZnO NPs (101.2%) exhibit the least induction among the synthesised ZnO-NPs groups, followed by ROT+UD-ZnO (130.9%) and ROT+MC-ZnO (190.2%). Plant extracts like ROT+UD (140.3%), ROT+MC (306.2%), and ROT+MK (121.3%) also inhibited mRNA expression.

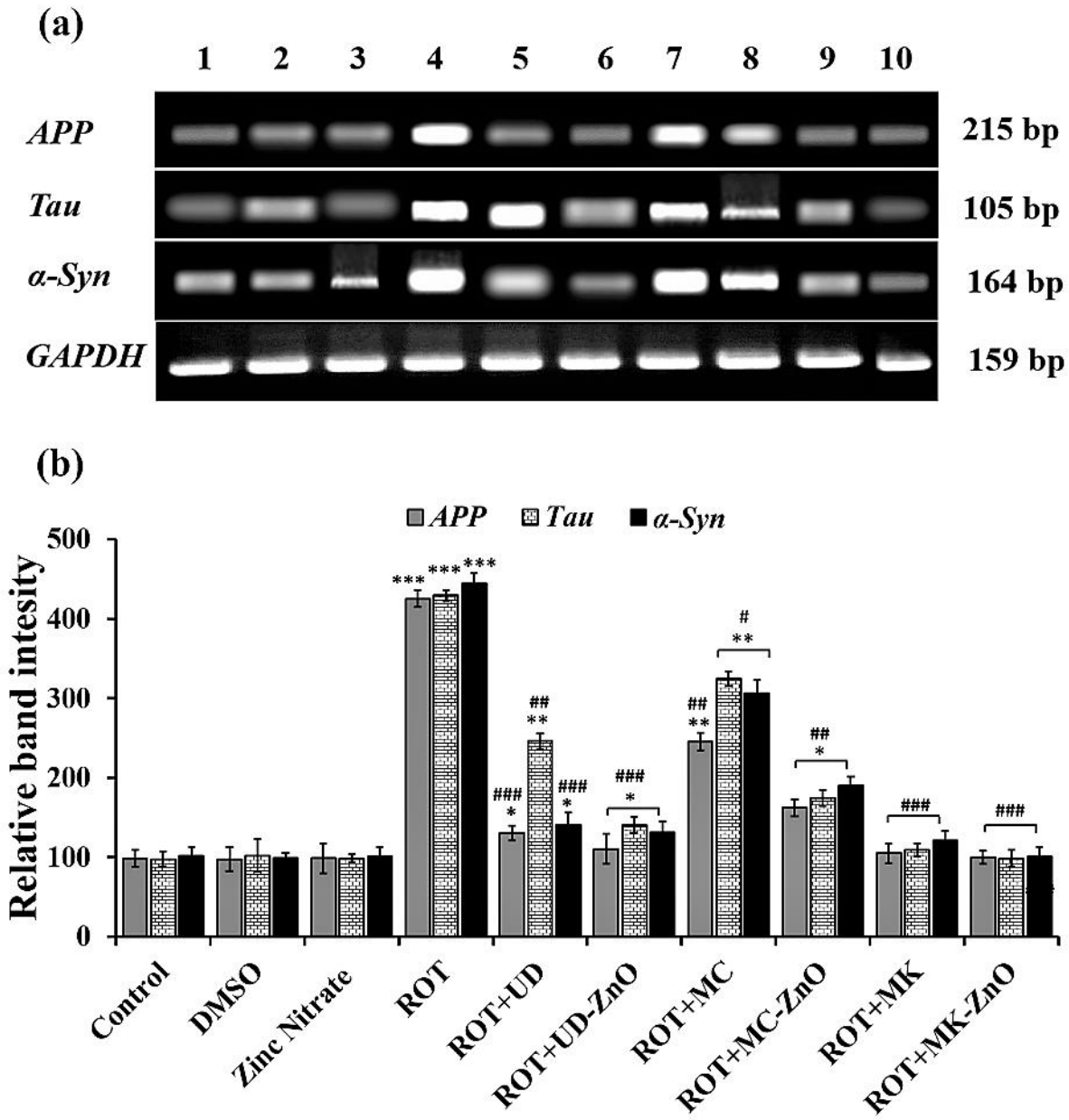


Figure 6.24: RT-PCR analysis of *APP*, *Tau*, and *α-Syn* mRNA in third instar larvae of *Drosophila melanogaster* (Oregon R^+) exposed to ROT (500 µM) alone or in association with UD-ZnO, MC-ZnO, and MK-ZnO for 48 h. Panel (a) represents an agarose gel picture from one of the experiments. Lane 1 (control), lane 2 (DMSO), lane 3 (Zinc Nitrate), lane 4 (Rotenone), lane 5 (ROT+UD), lane 6 (ROT+UD-ZnO NPs), lane 7 (ROT+MC), lane 8 (ROT+MC-ZnO NPs), lane 9 (ROT+MK), lane 10 (ROT+MK-ZnO NPs), respectively. Panel (b) represents the quantification of *APP*, *Tau*, and *α-Syn* mRNA expression levels (%) normalized to *GAPDH*. Data represent mean±SD (n=3); **P*<0.05, ***P*<0.01, ****P*<0.001 vs. untreated/control, DMSO or zinc nitrate control. # ascribed as significance at *P*<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$< 0.001 compared with 500 µM of rotenone. Note: ROT (Rotenone), UD (*Urtica dioica* extract), UD-ZnO (*Urtica dioica* synthesized Zinc oxide NPs), MC (*Matricaria chamomilla* extract), MC-ZnO (*Matricaria chamomilla* synthesized Zinc oxide NPs), MK (*Murraya koenigii* extract), MK-ZnO = *Murraya koenigii* synthesized Zinc oxide NPs.

Rotenone is a commonly used neurotoxin that causes pathological alterations which leads to mitochondrial dysfunction, oxidative stress, and neuronal damage, mimicking the features observed in neurodegeneration. Phyto-synthesized ZnO nanoparticles have been studied for their ability to inhibit the aggregation of Aβ, which is a key pathological feature of AD. These nanoparticles can interact with Aβ peptides, preventing their self-assembly into toxic oligomers and fibrils. This inhibition of Aβ aggregation can help reduce neuronal toxicity and synaptic dysfunction associated with AD. Tauopathies, including certain forms of dementia and frontotemporal dementia, are characterized by the abnormal aggregation and accumulation of hyperphosphorylated tau protein. Bio-synthesized ZnO nanoparticles demonstrated promise in reducing tau phosphorylation and stabilizing tau protein structure. These nanoparticles can potentially interfere with the pathological processes leading to tau aggregation, thereby mitigating neuronal damage and cognitive decline. PD is characterized by the degeneration of dopaminergic neurons and the presence of Lewy bodies, which are primarily composed of aggregated *α-synuclein*. Bioengineered ZnO nanoparticles have demonstrated anti-aggregation properties against α-synuclein, inhibiting its fibrillation and reducing cytotoxicity. These nanoparticles can also alleviate rotenone-induced oxidative stress and mitochondrial dysfunction, which are implicated in PD pathology.

Overall, phyto-synthesized ZnO nanoparticles hold promise as potential neuroprotective agents against alterations caused by rotenone in neurodegeneration. These nanoparticles, due to their unique properties, can scavenge free radicals and reduce oxidative stress in neuronal cells. However, the research in this field is still evolving, and further studies are needed to better understand the mechanisms involved and assess the long-term safety and efficacy of these nanoparticles for neuroprotection.

CHAPTER-7

DISCUSSION

This study demonstrated the neuroprotective properties of green engineered zinc oxide nanoparticles (ZnO-NPs) of *Urtica dioica* (UD-ZnO), *Matricaria chamomilla* (MC-ZnO), and *Murraya koenigii* (MK-ZnO) against rotenone-induced toxicities for the first time in a nontarget organism, *D. melanogaster*.

Systemic administration of drug to the CNS (central nervous system) is a substantial obstacle, owing to their short half-life, considerable first-pass digestion, restricted accessibility to the brain, and potential adverse impact when accessing non-target peripheral organs (Mansor et al., 2019). As a result, developing systemic delivery mechanisms with higher potency is critical for CNS pharmacotherapy. Cholinesterase (ChE) inhibitors, tacrine, N-methyl-D-aspartate (NMDA) agonists in connection with Vitamin D, levodopa, or other dopaminergic agonists and memantine have never been utilized in conventional medicine or therapeutics to alleviate anything apart from motor symptoms by replenishing neurotransmitters (Nong et al., 2022). However, prolonged use of these medications can have substantial side effects, including other motor challenges (Nakmode et al., 2023). To stop or reduce the progression of numerous neurodegenerative disorders, researchers must create new natural neuroprotective agents due to the inadequacy of treatment medicines (Dunkel et al., 2012). By activating the Nrf2/Akt/PI3K cascade and eliminating free radicals, nutraceuticals and other phytonutrients have been proven to have protective benefits and to reduce the consequences of neurodegenerative conditions (Rahul and Siddique, 2021). It is noteworthy that the Ayurvedic medical system has well established benefits of UD, MC, and MK for improving cognition, memory, and learning. To our knowledge, this is the first study to compare the therapeutic value of plant extracts (UD, MC, and MK) and their biosynthesized ZnO-NPs against ROT-induced toxicities in *Drosophila*.

Plants possess a remarkable capacity to produce various compounds that act as antioxidants, helping to protect against reactive oxygen species. ROS are extremely reactive compounds that are produced as normal byproducts of cellular metabolism and can cause harm to cells and tissues if not properly managed (Pan et al., 2020). Antioxidants neutralize ROS by donating an electron or hydrogen atom, thereby stabilizing these reactive molecules and preventing them from causing oxidative damage (Diplock et al., 1988).

In this research, DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) *in vitro* assays were used to measure the antioxidant activity of aqueous and ethanolic fractions of UD, MC, and MK plant extracts. The capacity of antioxidants to scavenge free radicals and diminish the colour intensity of the DPPH or ABTS radical, demonstrating their antioxidant potential, was used in these experiments. The results demonstrated that the ABTS activity in MK extracts did not differ significantly from the free radical scavenging potential measured by the DPPH assay because both of these assays use the same mechanism (single-electron transfer). Among the three different aqueous extracts, MK extract displayed the greatest scavenging activity with lowest EC_{50} values in the DPPH radical scavenging assay and as well as in the ABTS radical scavenging assay followed by UD extract and MC extract, as shown in **Figure 6.1**.

In ethanolic extracts, the similar pattern was seen. Previous studies found that the MK fraction is high in phenolic components such as flavonoids, tannins, and phenolic acids. These chemicals have antioxidant capabilities and can more effectively scavenge free radicals (Mitra et al., 2012). In investigations, MK extract has been shown to improve the activity of natural antioxidant enzymes such as SOD and CAT in various tissues (Yankuzo et al., 2011). By eliminating toxic ROS, these enzymes play a crucial role in oxidative damage defence (Mani et al., 2013). Rutin, caffeic acid, chlorogenic acid, kaempferol, and quercetin are among the phenolic substances found in UD extract. These compounds have been shown to have antioxidant activity, which assists in eliminating free radicals and the decrease of oxidative damage. Earlier research revealed that the extract had significant antioxidant properties, as indicated by its ability to remove free radicals and prevent lipid peroxidation, a process that damages cell membranes (Vajic et al., 2018). Moreover, MC extract contains various bioactive compounds that contribute to its antioxidant properties, including flavonoids, terpenoids, and phenolic compounds. These compounds have been found to exhibit antioxidant effects in laboratory studies. The researchers discovered that the extract has strong antioxidant activities, as evidenced by the ameliorative effects of MC hydroalcoholic fraction on scopolamine exposed memory impairment in rats (Ionita et al., 2018).

Additionally, chamomile's antioxidant activity might attribute to its anti-inflammatory qualities. Oxidative stress and inflammation are closely linked, and antioxidants can help reduce inflammation by neutralizing free radicals and modulating inflammatory pathways (Ranjbar et al., 2014). Ethanolic extracts of UD, MC, and MK were more potent in antioxidant

activity than aqueous extracts. This could be due to the aqueous solvent and extraction method used. Ethanol is often more efficient at extracting certain bioactive compounds compared to water, especially lipophilic compounds that may not be easily extracted using aqueous solvents alone (Biswas et al., 2012).

Phenolic compounds are a diverse collection of chemical molecules with antioxidant characteristics. They are plentiful in plants and have been linked to a number of health advantages. Flavonoids are phenolic components that have anticancer, anti-inflammatory, and antioxidant activities (Razem et al., 2022). Total flavonoid content (TFC) and total phenolic content (TPC) assays were performed to evaluate the overall concentration of phenolic components and flavonoids in a given sample. In both aqueous and ethanolic fractions, the total concentration of TPC and TFC were highest in MK, followed by UD, and MC extract had the lowest TPC and TFC values. This could be due to the extraction solvent used, as ethanolic extraction solvent was previously regarded to be the best for extracting total flavonoids (Stalikas, 2007). UD has been extensively studied and has shown prominent results in the treatment of prostate enlargement (Bhusal et al., 2022), in preventing colon carcinogenesis in rats (Uyar et al., 2022), and in providing a protective effect against hyperglycemia (Chehri et al., 2022), hypertension (Ahmadipour and Khajali, 2019) and hypercholesterolemia (Samakar and Hosseinzadeh, 2022). Numerous studies have shown that MC counteracts diabetes by controlling GLP-1, which is essential for promoting insulin gene transcription (Perestrelo et al., 2022), and it might be used to treat lung cancer (Aida et al., 2021). Supplementation of MK leaves have been found to have a significant preventive effect against TCA-induced liver injury (Aniqa et al., 2022) and lung cancer (Mondal et al., 2022). Additionally, earlier research on the MK leaf fraction supports the finding of efficacy in the management of hyperglycemia (Rajkumar and Vinotha, 2022). Although UD, MC, and MK are used for some *in vitro* and *in vivo* investigations, there is little evidence for the effect of these three herbs on cellular and neurological complications.

UV-Visible spectroscopy is an extensively utilised method to analyze the absorption and transmission of light in the ultraviolet (UV) and visible (Vis) regions of the electromagnetic spectrum by a substance, such as a plant extract. UV-Vis spectrum analysis was performed at intervals of 1 nm from 200 to 800 nm and revealed maximal absorption at 284 and 331 nm for UD, 267 and 326 nm for MC, and 286 and 317 nm for MK. The obtained UV-Visible spectrum of the plant extract represents the absorption or transmission pattern of light as a function of

wavelength. Different compounds present in the extract may exhibit characteristic absorption bands or peaks at specific wavelengths, allowing for the identification and analysis of those compounds (Zavoi et al., 2011). Peaks in crude extracts indicate the presence of a variety of ingredients or chemicals, particularly unsaturated bonds in conjugated or aromatic components. For example, various pigments, such as chlorophylls and carotenoids, present in plant extracts may exhibit distinctive absorption peaks in the visible region of the spectrum (Wyrostek et al., 2020).

The chemical structures of the bioactive elements in the plant fractions were determined using a fourier transform infrared spectrometer. In accordance with the peak intensity in the infrared radiation (IR) region. The specific wavenumbers and intensities were determined in the range of 4000-400 cm-^{1} (Al-Tameme et al., 2015). Both stretching and bending vibration assignments were compared to data from the literature. **Figure 6.5** represents the FTIR spectrum of UD, MC, and MK extracts in the form of KBr pellets and shows the presence of phenols, alcohols, ketones, nitro compounds, esters, carboxylic acids, ethers, aliphatic fluoro, alkenes, and aromatic rings. The broad absorption bands observed at 3358.28, 3327.40, and 3307.88 cm^{-1} can be attributed to the stretching of hydroxyl groups and H-bonding in alcohol or phenol groups (Qais et al., 2019). The weak absorption peaks in alkanes are detected at 2923.25, 2921.90, and 2974.04 cm^{-1}, which correspond to C–H stretching. N–H bends in primary amines are indicated by the high absorption peaks at 1698.70, 1622.77, 1601.79, and 1611.44 cm^{-1}. C–C stretching in aromatic groups is assigned to the medium peaks at 1455.85 and 1404.99 cm^{-1}. The rocking of the methyl group was assigned to the vibrational absorption bands at 1399.89 and 1396.51 cm^{-1}. C-O stretching is represented by distinct bands at 1271.82 and 1275.04 cm^{-1}. The C–N stretch in aliphatic amines is assigned to the thin peaks at 1055.84, 1074.42, 1043.61, and 1044.62 cm^{-1}. The aromatic H out-of-plane bending has bands at 877.47, 720.06, 659.68, and 620.82 cm^{-1} (Jiang et al., 2016). The obtained FTIR spectrum of the plant extract represents the absorption or transmission of infrared light as a function of wavelength or wavenumber. Different functional groups and chemical bonds present in the extract can absorb infrared light at characteristic frequencies, leading to specific peaks or bands in the spectrum. These peaks provide information about the chemical composition and molecular structure of the compounds present in the plant extract.

HPLC is a widely used analytical technique for separating, identifying, and quantifying components in a plant extract. The existence of 25 chemicals in the ethanolic fractions of UD,

MC, and MK was identified using HPLC. In the UD ethanolic extract, three classes of phenols were characterized: anthocyanin compounds (rosinidin 3-O-rutinoside; peonidin 3-O-rutinoside and peonidin 3-O-(6′-O-p-coumaroyl glucoside)**,** hydroxycinnamic acid derivatives (p-coumaric acid; chlorogenic acid; caffeoylquinic acid; 2-O-caffeoylmalic acid), and flavonoids (rutin; isorhamnetin 3-O-rutinoside; kaempferol 3-O-glucoside; quercetin; p-coumaroyl glucoside; kaempferol 3-O-rutinoside and quercetin 3-O-glucoside) as described earlier (Pinelli et al., 2008). The polyphenolic compounds found in the ethanolic fractions of MC flowers were identified by comparison with a previous document (Tsivelika et al., 2021). Our findings showed the existence of important constituents such as quercetin (quercetin-7-O-β-glucoside; quercetin-3-O-β-rutinoside and quercetin-3-O-β-galactoside), apigenin (apigenin-7-O-7-glucoside; apigenin-7-O-apiosyl-glucoside and apigenin-7-O-glucosyl-6'-acetate), luteolin (luteolin-7-O-β-glucoside; luteolin-4'-O-7-β-glucoside and luteolin-7-O-β-rutinoside), isorhamnetin (isorhamnetin-7-O-β-glucoside), patuletin (patuletin-7-O-β-glucoside), eupatoletin, astragalin, chrysosplenol and spinacetin (Nagappan *et al*., 2011). The MK ethanolic fraction was examined by HPLC-DAD, which permitted the identification of important components such as chlorogenic acid, quercitrin, citric acid, piperine, 7 p-coumaric acid, hesperidin, rutin, gallic acid, β-terpineol, ferulic acid, catechin, naringenin, D-α-pinene, di-α-phellandrene, dipentene, D-sabinene, caryophyllene, nicotinic acid, koenigine-quinone A and koenigine-quinone B (Ningappa and Srinivas, 2008). The HPLC data, including peak areas, retention times, and calibration curves, used for qualitative analysis of the plant extracts. Identification of compounds was done comparing retention times and spectral data with reference standards (rutin and quercetin) and published literature.

Drug targeting and delivery of dietary antioxidants to the brain provides significant obstacles due to the BBB (blood-brain barrier) in treating oxidative stress-related disorders, particularly neurodegenerative diseases (Grewal et al., 2017). Therefore, we employed innovative nanotechnology-based method, such as nanoparticles as nanocarriers, to cross the BBB and deliver the proper dosage of drug/medicine to target brain area.

Pure ZnO-NPs of high grade were synthesized by a completely green chemistry approach using UD (leaves), MC (flowers), and MK (leaves) fractions as a reducing agent and zinc nitrate hexahydrate ($Zn(NO_3)_2$) as the precursor. The green fabrication mechanism for the generation of ZnO nanoparticles based on plant phytochemicals that can function as capping and reducing agents to convert metal precursors into metal NPs (Ahmed et al., 2017). Phytochemicals,

although being antioxidants and non-toxic substances, may act as stabilising agents (Basnet et al., 2018). The major phytochemicals including methylxanthines, alkaloids, aldehydes, terpenoids, flavonoids, and phenolic acids that have attributed to the process of reduction. These phytoconstituents capping/reducing agents are found in variable amounts in diverse plant extracts (Aldalbahi et al., 2020). As a result, the content of plant extract has a substantial impact on the synthesis of NPs. Temperature, pH, metal salt concentration, contact duration, and phytochemical profile of plant extract all influence the production, stability, and formation of NPs. In order to stabilize the metal ions after being reduced by plant extracts, they will be encapsulated as an organic coating in three processes (Alhujaily et al., 2022). The activation phase involves reduction of metal ion and the nucleation of reduced metal ions. The growing phase helps to keep nanoparticles stable. The growth phase contributes to the stability of nanoparticles. The NPs shapes are determined in the termination phase. Metal ions enter the phase of growth and stabilization through the action of phytochemicals (Agarwal and Shanmugam, 2020).

The ZnO-NPs were fabricated in the form of pale yellow paste, which were calcinated at 400˚C to form powder and used for characterization. Their optical and structural properties were characterized through UV-Vis, FT-IR, XRD, DLS, EDS, SEM, Zeta size and potential in order to determine the agglomerated crystalline and hexagonal-shaped structure. The following techniques were used to analyze ZnO-NPs.

The stability and synthesis of bio fabricated ZnO-NPs were confirmed by UV-visible spectroscopy investigation. The samples were dissolved in deionized water for this analysis. The UV-visible spectrum ranged from 200 to 800 nm. The absorption peaks for UD-ZnO, MC-ZnO, and MK-ZnO NPs were obtained at wavelengths of 308, 319, and 305 nm, respectively. Barzinjy et al. (2020) claim that when the size of the NPs decreases, the absorption edge constantly shifts to a higher energy or lower wavelength. Previously, the peaks at 289-385 nm were recognised as the surface plasmon resonance of ZnO nanoparticles. The energy band gap is calculated from the UV-Vis graphs of UD-ZnO, MC-ZnO, and MK-ZnO NPs as 4.0, 3.8, and 4.0eV, respectively. The mobility of the electronic cloud on the overall structure of the ZnO-NPs may be responsible for the wide absorption band that extends towards longer wavelengths (Selim et al., 2020). The plant extract was also subjected to UV-vis analysis, and the findings revealed several peaks at various wavelengths between 200 and 350 nm. The plant fractions were found to contain proteins, reducing sugars and antioxidant molecules. These findings are

strikingly analogous to previous study (Al-Dhabi et al., 2018). In general, the considerably quick colour change and acute absorbance intensity shown in the initial few minutes of the procedure demonstrate the capabilities of the UD, MC, and MK fractions in ultra-fast nanoparticle production.

The crystalline structure and phase fraction analyses of biogenic ZnO-NPs were determined using the X-ray diffraction method (Kiani et al., 2022). The diffraction pattern's peak locations were utilized to calculate the translational symmetry-size and shape of the unit cell. The diffraction peaks of biogenic ZnO-NPs were obtained for 2θ values at 31.7, 34.5, 36.4, 47.8, 56.2, 62.5, 66.2, 67.6, 69.1, 72.9 and 77.2 corresponds to the plane of reflections for the values of (100), (002), (101), (102), (110), (103), (200), (112), (201), (004), and (202), respectively. These results exhibit distinct, strong peaks, which denotes that the biosynthesized ZnO-NPs are very pure and crystalline. The high intensity peak of ZnO-NPs from the XRD pattern at 101 planes is recognised as the characteristic peak of UD-ZnO, MC-ZnO, and MK-ZnO NPs after comparison with the standard powder diffraction card of JCPDS. The purity of the synthesised ZnO-NPs is proven by the absence of additional diffraction peaks. Hence, the XRD analysis demonstrated that the reduction of metal ions by UD, MC, and MK plant fraction could result in the fabrication of ZnO-NPs with well-defined dimensions. Using the Debye-Scherrer equation, the average size of the biosynthesized UD-ZnO, MC-ZnO, and MK-ZnO NPs was determined as 42, 45, and 41 nm, respectively, which revealed that the nanoparticle has a spherical crystal structure. Earlier investigations on the green production of zinc oxide nanoparticles yielded similar X-ray diffraction patterns (Sana et al., 2020).

The existence of bioactive functional groups involved in the conversion of $Zn(NO_3)_2$ to ZnO-NPs was verified using FT-IR spectroscopy (Naseer et al., 2020). The results of the FTIR analysis suggest that the surface of the nanoparticles includes phytoconstituents such as glycosides, alkaloids, tannins, reducing sugars, ursolic acid, flavonoids, phenols, and others. The presence of phenolic and hydroxyl groups was suggested by the existence of wide energy bands between 3500-3200 cm^{-1}, which are related to stretching of O-H group vibrations. The minor intensity peaks in the 2940-2970 cm^{-1} region might be attributed to stretching C-H alkaline vibrations, as well as other potential molecules. The bands detected at 2300-2370 cm^{-1} might be explained by -C- stretching. There is a discernible N=O vibration in the 1660-1600 cm^{-1} range. Absorption in this area, on the other hand, might indicate the existence of carbonyl groups. The energy bands seen at 1190-1100 cm^{-1} might be amine stretching. Si-o-Si stretching

protein vibrations might be responsible for the bands seen between 1096 and 1010 cm^{-1}.The bands might be corresponded to a secondary amine activity at 867-800 cm^{-1}. In addition, the weak energy bands that were caused by the stretching of the ZnO molecules at 697.28, 542.35, 532.95 cm^{-1} allowed for greater detection of the generation of UD-ZnO, MC-ZnO, and MK-ZnO NPs, respectively. The area that corresponds to metal-oxygen is between 400 and 600 cm^{-1}. Consequently, it can be concluded that reduction mechanism is responsible for the major chemical processes associated in the production of ZnO-NPs utilizing the aqueous extracts of UD, MC, and MK. Enzymes and phytochemicals found in biological materials have a contribution in converting metal compounds into nanoparticles (Cheema et al., 2022). These findings correlates to previously published report of ZnO NPs synthesis using leaf fraction of Olive (Hashemi et al., 2016).

The stability of nanoparticles is a significant factor in determining their performance and potential applications. The long-term stability of phytosynthesized ZnO-NPs under varied fluctuation conditions was examined. Various spectrophotometric analysis revealed that the synthesis rate is directly proportional to the freshness of the sample and the amount of ZnO-NPs solution. SEM revealed that a pH range of 8-10 produced tiny, stable particles with diameters ranging from 10 to 60 nm. The acidic pH of the reaction, on the other hand, produced minimally stable particles of uneven form and size, which lost their integrity on the tenth day of the experiment. In addition, a storage temperature of ≤5°C gives the optimum long-term stability. Even after more than 2 months, the ZnO-NPs remained spherical, ultrafine (5-20 nm), and stable without agglomeration.

The size of the produced ZnO-NPs was measured using DLS. The particle size of bio-fabricated ZnO-NPs varied from 10.63 nm to 90.37 nm, with an average size of 43.31 nm. The greatest and smallest sizes of the NPs were separated by a gap of 45.18 nm, showing that the distribution of ZnO-NPs was restricted. Furthermore, the zeta potential value was utilised to assess the nanoparticles' stability. It was discovered to be -19.2, -17.4, and -18.5 mV for UD-ZnO, MC-ZnO, and MK-ZnO NPs, respectively, indicating excellent stability. The ZnO-NPs have a negative surface charge because *U. dioica, M. chamomilla*, and *M. koenigii* extract compounds have a strong binding affinity for them, which increases their stability and reduces their tendency to aggregate. The finding coincides with the results that have previously been published (Barzinjy et al., 2020; Alyamani et al., 2021).

SEM is a surface optical analysis that can evaluate different particle size, formation, shape, density, and surface morphology of fabricated NPs at the nano and micro sizes (10^{-9} and 10^{-6}, respectively) (Jones, 2012). According to the SEM pictures, some of the ZnO-NPs formed had spherical and hexagonal shapes, while others had irregular shapes. The ZnO-NPs were formed in a variety of shapes, some of which were spherical and hexagonal at different temperatures as shown in the SEM images and others of which were irregular. Different magnifications were used to examine the FE-SEM images. UD-ZnO, MC-ZnO, and MK-ZnO NPs have sizes ranging from 20 to 50 nm. The uneven shape of ZnO NPs may have been influenced by pH, temperature, and $Zn(NO_3)_2$ concentration (Gur et al., 2022).

Energy Dispersive X-Ray Spectroscopy was used to determine the elemental composition of ZnO-NPs. Each element has a distinct atomic structure, which results in distinct peaks on the X-ray spectrum. On the EDX, the chemical component of the plant extract utilized as a reduction agent is the source of other elements such as oxygen and carbon. Zinc emits a strong signal in the EDX spectrum, while Cu, O, and K elements emit weak signals. These weak signals are the result of macromolecules such as enzymes, protein, and carbohydrates found in the plant fraction's cell wall emitting X-rays (Umar et al., 2018).

The antioxidant abilities of green engineered UD-ZnO, MC-ZnO, and MK-ZnO NPs and their plant fractions were evaluated using the ABTS and DPPH free radical scavenging tests (Khaleghi et al., 2022). At different concentrations 0.1, 0.2, and 0.3 mg/mL the antioxidant potential was assessed. In case of DPPH, MK-ZnO NPs (72.3-92.5%) exhibited a greater capacity to scavenge free radicals than the UD-ZnO (68.4-81.9%) and MC-ZnO (54.1-72.7%) NPs. The ABTS test revealed a similar pattern, with biosynthesized MK-ZnO NPs (71.9-93.4%) followed by UD-ZnO (64.6-84.5%) and MC-ZnO (52.7-78.9%) in terms of their ability to scavenge free radicals. ZnO-NPs from all three plants demonstrated the highest scavenging efficiency when compared to their aqueous extracts. The findings are contradictory since some studies found higher antioxidant activity in ZnO-NPs while others found the reverse. Given the huge number of research reporting both types of results, both outcomes seem possible. The finding suggests that the ABTS and DPPH radical scavenging activities were dose-dependent as the concentration of biosynthesized ZnO-NPs increased, scavenging activities against both radicals were also increased (Tettey and Shin, 2019).

To observe the anti-bacterial effects of biosynthesized ZnO-NPs, an experiment on *E. coli* and *S. aureus* was carried out. The zone of inhibition of UD-ZnO, MC-ZnO, and MK-ZnO NPs and

their efficiency in comparison to antibiotic (chloramphenicol) were the major considerations. The maximum inhibition zone of biosynthesized ZnO-NPs in case of *E. coli* were observed in MK-ZnO NPs (1.5 cm) followed by UD-ZnO NPs (1.2 cm), and MC-ZnO NPs (1.0 cm). The similar pattern was observed in case of *S. aureus*, highest zone of inhibition was recorded in MK-ZnO NPs (1.7 cm) followed by UD-ZnO NPs (1.4 cm), and MC-ZnO NPs (1.4 cm). Although the mechanisms for delivering NPs into microbial cells have been identified, more research is still needed to determine exactly how nanoparticles interact with membrane receptors and efflux pumps to start antimicrobial activity (Happy et al., 2018). NPs can enter a cell in four ways: adhering to the membrane, destroying microbial DNA through the generation of ROS from Zn ions, tampering with ATP synthesis and replication of DNA from ionic forms of particles, and eventually creating thiols or phosphates from interactions with nucleic acid and amino acid moieties (Raghupathi et al., 2011). Nevertheless, the principal method through which ZnO acts with bacteria involves interfering with both capsule biosynthesis and central carbon metabolism. Greater zinc concentrations of ions in nanoforms reduce virulence by reducing the synthesis of hyaluronic acid capsules, which in turn inhibits numerous key enzymes that breakdown glucose and affects the development of carbon catabolic pathways (Aldeen et al., 2022). The green fabricated ZnO-NPs can be used for both diagnostic and therapeutic purposes for a variety of pathological diseases because to their promising properties. The destructive activity of UD-ZnO, MC-ZnO, and MK-ZnO NPs at minimum inhibitory levels for bacteria is most likely caused by its ability to damage cell membrane structures and permeability barriers with loss of chemiosmotic control. The finding corelates with the results that have previously been published (Azimpanah et al., 2022).

Considering the general protective and cognitive effects of biosynthesized ZnO-NPs through UD, MC, and MK extracts that have been reported in the literature. In the present study, third instar larvae and freshly eclosed flies were treated with 500 µM ROT alone or in combination with biosynthesized ZnO-NPs and their UD, MC, and MK extracts for 24 h to 120 h. Following exposure, cytotoxicity assay (dye exclusion test), neurochemical assays (acetylcholinesterase and butrylcholinesterase inhibition assays), biochemical assays (CAT, SOD, LPO, PC, protein estimation and GSH), behavioral assays (climbing, memory, jumping and crawling assays) and molecular parameter (RT-PCR) were performed. ROT is a well-known generator of ROS, which cause cellular damage and eventually cause necrosis or programmed cell death. Due to a lack of an effective antioxidant system, the cell would not be able to handle the harm caused by ROS. *L*-DOPA appears to simply act as a dopamine precursor to restore endogenous

dopamine deficits, as previous studies have shown that feeding the drug to flies did not reduce cell loss (Javed et al., 2020).

The dye exclusion test using trypan blue is a commonly used method to assess cell viability by distinguishing between live and dead cells. In the context, cytotoxicity of rotenone and its amelioration through green engineered ZnO-NPs was determined by dye exclusion test in whole larvae and tissues of ROT-exposed organisms. In 96% of the larvae treated to ROT, the whole larva and its tissues exhibited blue staining (salivary gland, midgut, brain ganglia, malpighian tubules, and gastric caeca). ROT co-exposed with MK-ZnO NPs demonstrated significantly less blue staining in the tissues mentioned above and the whole larvae when compared to ROT plus UD-ZnO and ROT plus MC-ZnO NPs groups, respectively. This conclusion is validated by a previous investigation that showed plant biomolecules significantly enhanced cell viability and attenuated DNA damage (Siima et al., 2020). Biogenic ZnO-NPs are protective due to the activation of antioxidant defence mechanisms or the presence of bioactive molecules that quench free radicals (Sehgal et al., 2011).

The trypan blue staining principle is based on the differential permeability of the cell membrane in living and dead cells. Trypan blue is a water-soluble dye that cannot penetrate living cells' undamaged cell membranes. When the trypan blue solution is introduced to the cell suspension, the dye stays outside the cells and the stain is not taken up. As a result, when observed using a microscope, living cells seem translucent or with a light refractive appearance. However, the cell membrane loses its integrity in dead or injured cells, enabling trypan blue to enter the cell and stain its contents. The dye subsequently attaches to numerous internal components including proteins and nucleic acids, giving the cell a blue colour. Under the microscope, dead or non-viable cells show as blue-stained structures.

Acetylcholinesterase (AChE) is a crucial enzyme of the cholinergic system that regulates physiological functions, including movement and memory (Chopra et al., 2022). By hydrolysing acetylcholine into choline and acetate, it inhibits cholinergic neurotransmission between synapses (Singh et al., 2022). In this study, it was observed that when the *Drosophila* larvae exposed to ROT for 24 hours showed a statistically significant (P<0.001) decrease in acetylcholinesterase activity compared to either DMSO or control, had ~68.91% reduced AChE levels. The level of AChE was enhanced when ROT was coexposed with MK-ZnO NPs and only 20.65% inhibition was evident. Comparing the ROT+UD-ZnO and ROT+MC-ZnO NPs groups to the control, the improvement in AChE levels was less prominent in both. After 48

hours, ROT alone exposed organisms had the highest amount of AChE inhibition compared to control larvae and the highest level of rescue from ROT-induced toxicity was seen in the ROT+MK-ZnO NPs group. Additionally, the ROT+UD-ZnO and ROT+MC-ZnO groups significantly had greater AChE levels than the ROT-treated group. According to obtained results, it suggests that the biosynthesized ZnO nanoparticles effectively enhanced the activity of AChE in *Drosophila melanogaster* that were exposed to rotenone. This implies that the phytosynthesized ZnO-NPs may have a protective effect on the nervous system by counteracting the inhibitory effects of rotenone on AChE activity, which is consisted with earlier studies (Ullah et al., 2022). Both plant-synthesized ZnO nanoparticles and plant extracts have the potential to rescue AChE inhibition, the effectiveness of each approach can vary depending on the specific application, the plant species involved, and the characteristics of the nanoparticles or extracts used (Akintunde et al., 2021).

Butyrylcholinesterase is an enzyme found in the bloodstream and is responsible for the breakdown of certain chemicals called cholinesterase inhibitors, including acetylcholine (Singh et al., 2022). In this study, third instar larvae of Oregon R^+ exposed to ROT for 24 hours revealed a statistically significant (P<0.001) reduction in butyrylcholinesterase activity as compared to either DMSO or control, with 66.31% lower BChE levels in the aforementioned group. When ROT and MK-ZnO NPs were co-exposed, these greater levels of butyrylcholinesterase were obviously significant when compared to ROT alone treated groups, with just 24.65% inhibition compared to control. Surprisingly, BChE levels improved significantly in the ROT plus UD-ZnO and ROT plus MC-ZnO NPs groups. Similar trend was observed after 48 hours, ROT-exposed organisms had the highest level of BChE inhibition compared to control/untreated larvae, whereas ROT + MK-ZnO NPs had the highest level of rescue from ROT-induced neurotoxicity followed by ROT plus UD-ZnO and ROT plus MC-ZnO groups. Plant extracts also showed promising results against rotenone induced butyrylcholinesterase inhibition. Based on the obtained observations, it suggests that when rotenone was used to inhibit BChE activity, the presence of plant-synthesized ZnO nanoparticles enhanced the activity of BChE. This enhancement might imply that the ZnO-NPs have a protective or restorative effect on the BChE enzyme, potentially mitigating the inhibitory effects of rotenone (Akintunde et al., 2021).

To understand the adverse consequences of rotenone, biochemical investigations were performed after 24 and 48 hours of administration. Rotenone is an inhibitor of mitochondrial

complex I that causes oxidative damage and affects the antioxidant defence mechanism. This can boost ROS levels and CAT activity, which is responsible for converting hydrogen peroxide (H_2O_2) into water (H_2O) and molecular oxygen (O_2). To investigate whether green ZnO-NPs could mitigate the rotenone-induced alterations in CAT assay were carried out in *Drosophila*. The catalase activity in the tissues of third instar larvae of *Drosophila* exposed to ROT (500 µM) increased statistically significantly (P<0.001). When compared to the control group, the catalase activity of the larvae in the ROT group was increased after 24 hours. The lowest CAT activity was observed in ROT co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs, and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) also shown promising effects in suppressing rotenone-induced CAT activity. Similar trend was observed after 48 hours, ROT co-exposed with MK-ZnO NPs showed a decrease in CAT activity, followed by ROT+UD-ZnO, and ROT+MC-ZnO NPs **(Figure 6.18-a)**. UD, MC, and MK extracts have been found to regulate the expression of genes involved in antioxidant defense, including CAT. This modulation of gene expression can help to normalize CAT activity in the presence of rotenone. Based on the data analysis, we conclude that green ZnO-NPs have a protective effect against rotenone-induced oxidative stress by restoring or enhancing the activity of these antioxidant enzymes. This outcome is consistent with earlier studies that showed pesticides decreased the CAT activities in organisms (Rahimi et al., 2022). Biosynthesized ZnO nanoparticles possess antioxidant properties due to the presence of bioactive compounds derived from plants. These nanoparticles can scavenge reactive oxygen species and reduce oxidative stress. By reducing the burden of ROS, biosynthesized ZnO nanoparticles may help preserve the activity of catalase, allowing it to function more efficiently in breaking down hydrogen peroxide.

The effects of green ZnO-NPs on rotenone-induced alterations to antioxidant enzymes, specifically SOD, in *D. melanogaster* have been studied. In this study, it was observed that SOD level increased considerably (P<0.05) in the tissues of third instar *Drosophila* larvae exposed to ROT (500 µM). After 24 hours, the SOD level of the larvae in the ROT group had risen than in the control group. ROT co-exposed with MK-ZnO NPs had the lowest SOD activity, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) have also been demonstrated to decrease rotenone-induced SOD activity. After 48 hours, the similar trend was observed when compared to the control group, ROT exposure resulted in a substantial increase in SOD level in the larvae. ROT co-exposed with MK-ZnO NPs reduced SOD expression, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs

(Figure 6.18-b). UD, MC, and MK extracts also have been reported to affect the expression of antioxidant defence genes such as SOD. Biosynthesized ZnO-NPs can potentially activate cellular defense mechanisms, including the antioxidant response pathway (Erfani et al., 2021). This activation can lead to the upregulation of SOD synthesis and increased levels of the enzyme in cells.

Lipid peroxidation is a process where ROS attack and damage lipids, leading to the generation of lipid peroxides. This assay helps assess oxidative stress and lipid damage in various biological systems. In this research, MDA level was significantly higher (P<0.001) in the tissues of third instar *Drosophila* larvae exposed to ROT (500 µM). After 24 hours, the MDA level in the ROT group was higher than in the control group. The lowest MDA concentration was found in ROT co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) reduced rotenone-induced MDA level as well. After 48 hours, when compared to the control group, ROT exposure resulted in a significant rise in MDA levels in the larvae. MDA expression was decreased when ROT was co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. UD, MC, and MK extracts all showed improved effects on the reduction of lipid peroxidation induced by rotenone. Plant-derived ZnO-NPs demonstrated the enhanced protective effect against lipid peroxidation. ZnO-NPs possess intrinsic antioxidant properties and can scavenge ROS. By reducing oxidative stress, these nanoparticles can help mitigate the generation of lipid peroxides, thereby lowering LPO levels. Phyto-synthesized nanoparticles can act as free radical scavengers, neutralizing reactive free radicals that contribute to lipid peroxidation (Bahr et al., 2020). By capturing and detoxifying these radicals, the nanoparticles can help protect lipids from oxidative damage and inhibit the process of lipid peroxidation.

Protein carbonylation occurs when reactive species attack proteins, leading to the formation of carbonyl groups on specific amino acid residues, such as lysine, arginine, proline, and threonine. The quantification of protein carbonyl content is a widely used assay to assess protein oxidation and oxidative stress levels in tissue homogenate. In this work, PC content was significantly increased (P<0.001) in the tissues of third instar larvae of *D. melanogaster* exposed to ROT (500 µM). After 24 hours, the PC concentration in the ROT group was higher than in the control group. The reduced PC concentration was found in ROT co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. Plant extracts (UD, MC, and MK) reduced rotenone-induced PC level as well. After 48 hours, when compared to

the control group, ROT exposure resulted in a significant rise in PC level in the larvae. PC expression was decreased when ROT was co-exposed with MK-ZnO NPs, followed by ROT+UD-ZnO NPs and ROT+MC-ZnO NPs. UD, MC, and MK extracts all showed improved effects on the reduction of protein carbonyl induced by rotenone. Green ZnO-NPs, with their intrinsic antioxidant properties, may have the potential to scavenge ROS and reduce oxidative stress (Sengani et al., 2023). This reduction in oxidative stress could potentially result in the decreased formation of protein carbonyls induced by rotenone.

Rotenone can affect dopaminergic neurons in *Drosophila*. Dopamine-related proteins, such as tyrosine hydroxylase (TH) or dopamine transporters, may be altered in response to rotenone exposure, potentially leading to changes in protein concentration. After 24 hours, the total protein concentration in the larval tissues (3rd instar) of *Drosophila* exposed to ROT (500 µM) decreased statistically significantly (p<0.001), when compared to the control and vehicular control (DMSO) groups. The highest protein content was determined in ROT co-exposed with MK-ZnO, followed by ROT plus UD-ZnO, and the least in ROT plus MC-ZnO. Similar pattern was observed after 48 hours, the ROT exposure has reduced the total protein concentration in the larvae compared to the untreated/control group. ROT co-exposed with MK-ZnO NPs showed an increase in protein concentration, then by ROT plus UD-ZnO, and ROT plus MC-ZnO NPs. This outcome is consistent with earlier studies that showed pesticides decreased the protein concentration in organisms (Pandareesh et al., 2016). The secondary metabolites found in plants that function as free radical scavengers, especially against oxygen radicals, and inhibit SH-group oxidation could be the protective mechanism of plant-derived ZnO-NPs (Gülçin et al., 2012).

The assay for reduced GSH is a commonly used method to measure the level of GSH, a vital antioxidant molecule, in tissue homogenate. GSH plays a crucial role in cellular defense against oxidative stress and is involved in various metabolic processes. The glutathione content in the tissues of third instar larvae of *Drosophila* exposed to ROT (500 µM) decreased statistically significantly (P<0.001). When compared to the control group, the GSH concentration of the larvae in the ROT group was reduced after 24 hours. Among the ZnO-NPs synthesized groups, the highest GSH content was observed in ROT co-exposed with MK synthesized ZnO NPs, followed by ROT plus UD-ZnO NPs, and the lowest in ROT plus MC-ZnO NPs. Plant extracts such as UD, MC, and MK also enhanced GSH concentration as well. After 48 hours, the ROT exposure has reduced the GSH content in the larvae compared to the control group. ROT co-

exposed with MK synthesized ZnO NPs showed an increase in GSH concentration, followed by ROT plus UD-ZnO, and ROT plus MC-ZnO NPs. This outcome is consistent with earlier studies that showed pesticides decreased the GSH concentration in organisms. Plant-synthesized ZnO nanoparticles exhibit antioxidant properties due to the presence of bioactive compounds derived from plants (Sehgal et al., 2012). These nanoparticles can scavenge harmful free radicals and reduce oxidative stress, which in turn can help preserve the level of GSH in the body.

The behaviour of an organism reflects its usual physiological function. The ability of an organism to climb can be indicative of its physical strength, coordination, and overall health. If an organism is experiencing physiological disruptions or impairment due to the presence of rotenone or any other factor, it may exhibit reduced climbing behavior. For example, if rotenone inhibits the organism's motor functions or causes muscle weakness, it may struggle to climb or show a decrease in climbing activity. In this study, the highest climbing potential was shown by the control, DMSO, and zinc nitrate-treated flies in 30 seconds. The maximum decrease was detected in ROT-treated *Drosophila*, and flies had trouble to climb the walls of cylinder. Different groups with ROT and plant-mediated ZnO-NPs demonstrate variable degrees of improvement in their climbing abilities. The ability of flies to ascend was increased by all green ZnO-NPs as illustrated in **Figure 6.21-a**. Among the synthesized ZnO-NPs groups, ROT plus MK-ZnO NPs exhibits the minimum reduction, followed by the ROT plus UD-ZnO and ROT plus MC-ZnO showed the greatest reduction in climbing activity. Obtained results suggests that plant extracts as well as their synthesized ZnO-NPs effectively protected flies from deteriorating locomotor dysfunctions, indicating that they might be able to defend by replenishing the dopamine pool at the mitochondrial level (Riemensperger et al., 2013). Green engineered ZnO-NPs modulated and enhanced neuromuscular function, improved muscle strength, coordination, and overall climbing performance.

Rotenone exposure has been associated with impaired memory function in various organisms. Memory deficits can manifest as difficulty in learning new tasks, impaired spatial memory, or reduced ability to recall previously learned information. Rotenone's impact on memory is thought to be related to its effects on the central nervous system, particularly the dopaminergic system and mitochondrial function. In this observation, the highest memory ability was shown by the control, DMSO, and zinc nitrate-treated flies. The greatest reduction was reported in ROT-treated *Drosophila*, and flies were observed moving randomly at the T-point of the

apparatus, and the flies were sluggish enough to reach the T-point. The ability of flies to memorize was increased by all green ZnO-NPs as illustrated in **Figure 6.21-b**. Among the synthesized ZnO-NPs groups, ROT plus MK-ZnO NPs exhibits the minimum reduction, followed by the ROT plus UD-ZnO and ROT plus MC-ZnO showed the greatest reduction in memory ability. Obtained results suggests that plant extracts as well as their synthesized ZnO-NPs enhanced memory in *Drosophila melanogaster* that had been exposed to rotenone. Plant-synthesized ZnO-NPs with antioxidant properties can scavenge harmful reactive oxygen species and reduce oxidative damage, potentially preserving neuronal health and supporting memory processes (Amara et al., 2015).

Jumping is a complex motor activity that requires coordination, muscle strength, and agility. Similar to climbing, an organism's ability to jump can reflect its physiological condition (Teixeira et al., 2022). If an organism is affected by rotenone, which can interfere with neuromuscular function, it may display reduced jumping ability or exhibit altered jumping patterns. The considerable reduction in jumping behaviour in exposed organisms, which was followed by an inhibition of AChE activity, demonstrated the pesticide's detrimental consequences on the organism. Poor locomotor activity has previously been identified as a symptom of AChE activity inhibition. The maximum rescue was demonstrated by ROT plus MK-ZnO, followed by ROT plus UD-ZnO and ROT plus MC-ZnO NPs. The observations support previous studies that plant nanoparticles have the capacity to biosynthesize several non-enzymatic antioxidants that can decrease ROS-induced oxidative damage (Ren et al., 2021). Plant-synthesized ZnO-NPs modulated and enhanced the signaling and connectivity at the neuromuscular junction, potentially improved jumping performance (Sehgal et al., 2013).

Crawling is a fundamental motor behavior, especially in early developmental stages of organisms. Rotenone exposure can disrupt motor coordination, muscle function, and neural pathways involved in controlling movement (Adedara et al., 2022). As a result, organisms exposed to rotenone may exhibit difficulty in crawling, such as reduced mobility, slower crawling speed, or impaired coordination of limb movements. The basic behaviour of *Drosophila* larval crawling enables us to examine the involvement of genes in the specification of neurons in the entire organism as well as the functions of those neurons. The control, DMSO, and zinc nitrate-treated larvae had the highest level of crawling activity in 1 minute. The largest reduction was seen in *Drosophila* treated with ROT, and flies had difficulty crawling on petri plates. Different ROT and plant-mediated ZnO-NP groups exhibit varying degrees of

improvement in their ability to crawl. All green ZnO-NPs improved the ability of larvae to crawl appropriately, as shown in **Figure 6.22-b**. In this study, it was observed that the crawling ability was highly rescued in ROT plus MK-ZnO NPs), followed by ROT plus UD-ZnO, and ROT plus MC-ZnO among the synthesised ZnO-NPs groups. Phytosynthesized ZnO-NPs possess unique properties that can be utilized for targeted delivery of therapeutic agents or enhancing the efficacy of crawling ability. Plant-synthesized ZnO-NPs modulated neural communication pathways and improved the connectivity and signaling within the nervous system (Bell et al., 2015). This potentially enhanced motor coordination and crawling ability (Nitta and Sugie, 2022).

To investigate whether there is any change in the size or shape of the *Drosophila* eyes treated with rotenone (500 µM) alone or in association with UD-ZnO, MC-ZnO, and MK-ZnO and their plant extracts for 48 h, eye imaging under stereo zoom microscope was done. In this study it was observed that the prominent dark area (degeneration) in ROT-treated *Drosophila* compared to control, DMSO, and zinc nitrate-treated flies which showed clear eyes. Different groups with ROT and plant-mediated ZnO-NPs demonstrate variable degrees of improvement as illustrated in **Figure 6.23**. Among the synthesized ZnO-NPs groups, ROT plus MK-ZnO exhibits the minimum degeneration, followed by the ROT plus UD-ZnO and ROT plus MC-ZnO showed the greatest eye degeneration. Plant-synthesized ZnO nanoparticles with antioxidant properties can scavenge harmful reactive oxygen species and protect cells from oxidative damage, potentially slowing down the degenerative process and preserving visual function. The observations support previous studies that plant based ZnO-NPs can potentially enhance tissue regeneration and repair in the eye (Varga et al., 2014). By stimulating cellular proliferation, migration, and differentiation, these nanoparticles may contribute to the restoration of damaged tissues and the recovery of visual function.

To further understand the underlying mechanisms of rotenone-induced degeneration, we performed molecular analyses, such as gene expression profiling, to identify changes in specific genes or signaling pathways associated with the degenerative process. RT-PCR (Reverse Transcription Polymerase Chain Reaction) was carried out to analyze the expression levels of genes such as tau, amyloid precursor protein, and α-synuclein. The dysregulation of *APP*, *tau*, and *α-Syn* expressions has been strongly implicated in the pathogenesis of neurological diseases (AD and PD) and can contribute to the process of neurodegeneration. The accumulation and aggregation of APP can lead to the formation of amyloid-β plaques, a

hallmark pathology in AD. Increased APP expression induced by rotenone suggests a significant role in promoting the amyloidogenic pathway and the progression of AD pathology (Yang et al., 2022). Rotenone exposure has also been associated with the upregulation and hyperphosphorylation of tau protein. Tau is normally involved in stabilizing microtubules in neurons, but abnormal phosphorylation can lead to the formation of NFTs, a characteristic feature of tauopathies such as AD (Iqbal et al., 2005). The upregulation of tau protein induced by rotenone suggests a potential contribution to tau pathology and neurodegeneration. Alpha-synuclein aggregates are a pathological hallmark of PD and other synucleinopathies (Xu and Pu, 2016). The upregulation of alpha-synuclein by rotenone suggests its involvement in the neurodegenerative processes associated with Parkinson's disease (Rocha et al., 2018).

RT-PCR gene expression studies was performed using *GAPDH* (159 bp) as the housekeeping gene, whereas *APP* (215 bp), *Tau* (105 bp), and *α-Syn* (164 bp) as the target genes. In case of *APP* gene, the maximum upregulation ($P<0.05$) was observed in larvae treated with ROT in comparison to control, DMSO, and zinc nitrate, respectively. Among the biosynthesized ZnO-NPs groups, ROT plus MK-ZnO NPs exhibits the minimum induction, followed by the ROT plus UD-ZnO and ROT plus MC-ZnO also showed the decrease in gene expression. Plant extracts such as ROT plus UD, ROT plus MC, and ROT plus MK suppressed the *APP* mRNA expression as well. In terms of *tau* gene, the greatest upregulation ($P<0.05$) was seen in larvae treated with ROT compared to control, DMSO, and zinc nitrate, respectively.

Among the synthesised ZnO-NPs groups, ROT plus MK-ZnO NPs demonstrated the least induction, followed by ROT plus UD-ZnO and ROT plus MC-ZnO. Plant extracts such as ROT plus UD, ROT plus MC, and ROT plus MK also downregulated the *tau* mRNA expression. In case of *α-Syn*, larvae treated with ROT demonstrated the maximum upregulation ($P<0.05$) compared to control, DMSO, and zinc nitrate, respectively. ROT plus MK-ZnO NPs exhibited the least induction among the synthesised ZnO-NPs groups, followed by ROT plus UD-ZnO and ROT plus MC-ZnO. Plant extracts like ROT plus UD, ROT plus MC, and ROT plus MK also inhibited mRNA expression. The results suggests that biosynthesized ZnO-NPs have demonstrated promise in modulating the expressions of *APP*, *tau*, and *α-Syn*, which are involved in neurodegenerative diseases. The finding coincides with the results that have previously been published (Kim et al., 2017). These nanoparticles may possess properties that inhibit the aggregation of *APP*, *tau*, and *α-Syn* proteins. By preventing or reducing the

formation of protein aggregates, these nanoparticles could potentially help decrease the expressions of these disease-related proteins.

Altogether, the present work showed the comparative efficacy of biosynthesized ZnO-NPs against microorganisms (*E. coli* and *S. aureus*), in addition to the nontarget organism *Drosophila* at neuronal, organismal, cellular, and molecular levels. The synthesized ZnO nanoparticles were analyzed using SEM, UV-Vis, XRD, DLS, EDX, FT-IR, and Zeta potential techniques. The bio-fabricated ZnO-NPs using extracts of the selected plants have been found to showed more intensified antioxidant and antibacterial properties as compared to plant extracts. A short-term dietary administration of UD-ZnO, MC-ZnO, and MK-ZnO NPs to *D. melanogaster* has the potential to reduce ROT-induced oxidative stress, improve locomotion, restore AChE levels, downregulated *APP*, *tau*, and *α-Syn* expressions provided as evidence of their neuroprotective properties. The limitations of conventional drug administration are dramatically improved by the green synthesis of ZnO-NPs with drug targeting, sustained release, and significantly increased bioavailability of drugs. More investigation is required to elucidate and mediate the mechanisms by which the blood-brain barrier is crossed, as well as to improve the efficiency of nanotechnology-based brain delivery techniques.

CHAPTER-8

SUMMARY AND CONCLUSION

The homeostasis among both antioxidants and oxidants regulates the redox status of cells. Any imbalance between these two that can result in necrosis or apoptosis determines the oxidative status of the cell. The key factor contributing to the increased oxidative stress sensitivity of brain cells is ROS. It leads to dreaded conditions including neurodegenerative diseases. Systemic administration of drug to the CNS is a substantial obstacle, owing to their short half-life, considerable first-pass digestion, restricted accessibility to the brain, and potential adverse impact when accessing non-target peripheral organs. As a result, developing systemic delivery mechanisms with higher potency is critical for CNS pharmacotherapy. Cholinesterase (ChE) inhibitors, tacrine, N-methyl-D-aspartate (NMDA) agonists in connection with Vitamin D, levodopa, or other dopaminergic agonists and memantine have never been utilized in conventional medicine or therapeutics to alleviate anything apart from motor symptoms by replenishing neurotransmitters. However, prolonged use of these medications can have substantial side effects, including other motor challenges. To stop or reduce the progression of numerous neurodegenerative disorders, researchers must create new natural neuroprotective agents due to the inadequacy of treatment medicines. By activating the Nrf2/Akt/PI3K cascade and eliminating free radicals, nutraceuticals and other phytonutrients have been proven to have protective benefits and to reduce the consequences of neurodegenerative conditions. The Ayurvedic medicinal system has long employed the plants UD (*Urtica dioica*), MC (*Matricaria chamomilla*), and MK (*Murraya koenigii*) as well-known nerve relaxants and cognition boosters. These three herbs include phytochemicals with proven protective properties, including quercetin, alkaloids, vitamins, and polyphenols. Drug targeting and delivery of dietary antioxidants to the brain provides significant obstacles due to the BBB in treating oxidative stress-related disorders, particularly neurodegenerative diseases. Novel approaches for the improved crossing of the BBB must be developed in order to progress in the effective therapies.

Nanotechnology holds significant ameliorative potential against neurodegenerative diseases as it can protect the therapeutic substance and allow for its sustained release. This study demonstrated that pure ZnO-NPs of magnificent quality were fabricated utilising zinc nitrate hexahydrate as the metal precursor and plant (UD, MC, and MK) extracts as the reducing agents against bacteria (*Staphylococcus aureus and Escherichia coli*) and against rotenone-induced toxicities in *D. melanogaster* for the first time. Their optical and structural properties

were analyzed through DLS, FT-IR, XRD, EDS, SEM, UV-Vis, and Zeta potential in order to evaluate the agglomerated crystalline and hexagonal-shaped structure with an average diameter of 20-40 nm. The antioxidant and antimicrobial properties of fabricated ZnO NPs were evaluated employing cell free models (DPPH and ABTS), and well diffusion method, respectively. The rotenone (500 µM) was administered to *Drosophila* third instar larvae, and freshly emerged flies for 24-120 hours, either alone or in combination with biogenic ZnO-NPs. A comparative study on the protective properties of synthesized NPs was undertaken against rotenone-induced toxicities. Following exposure, dye exclusion assay (cytotoxicity test), neurochemical assays (acetylcholinesterase and butrylcholinesterase inhibition analyses), biochemical assessments (CAT, SOD, LPO, PC, protein estimation and GSH), behavioral assays (climbing, memory, jumping and crawling assays) and molecular parameter (RT-PCR) were carried out. RT-PCR gene expression studies was performed using *GAPDH* (159 bp) as the housekeeping gene, whereas *APP* (215 bp), *Tau* (105 bp), and *α-Syn* (164 bp) as the target genes. Rotenone exposure has been associated with the upregulation of *APP, α-Syn,* and *tau* protein. The findings revealed that among the plant derived ZnO-NPs, MK-ZnO NPs exhibit strong anti-microbial and antioxidant activities followed by UD-ZnO NPs, and MC-ZnO NPs. In this regard, ethno-nano medicinal therapeutic uses mimic the similar effects in *D. melanogaster* by suppressing (p<0.05) oxidative stress and counteracting the inhibitory properties of rotenone on neurochemical activities (BChE and AChE activity). Biosynthesized ZnO-NPs have demonstrated tremendous effects in modulating the aggregates of *APP*, *tau*, and *α-Syn* expressions, which are involved in neurodegenerative pathologies. Plant-derived ZnO-NPs showed the enhanced protective effects against oxidative stress by restoring biochemical parameters (CAT, SOD, LPO, PC, GSH and proteotoxicity activity). By reducing cellular toxicity (trypan blue), these nanoparticles possess intrinsic antioxidant properties and can help mitigate the generation of ROS. ZnO-NPs effectively protected flies from deteriorating locomotor dysfunctions, indicating that they might be able to defend by replenishing the dopamine pool at the mitochondrial level. These nanoparticles modulated and enhanced neuromuscular function, improved muscle strength, coordination, supporting memory processes, and overall climbing performance. These findings suggest that green engineered ZnO-NPs have potential to significantly enhance outcomes, with the promise of effective neurodegenerative therapy and could be used as great alternative for clinical development.

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APPENDIX I:

LIST OF PUBLICATIONS AND PRESENTATIONS

APPENDIX 1 **LIST OF PUBLICATIONS**

S.NO.	TITLES	IMPACT FACTOR
1.	Ethnopharmacological Effects of *Urtica dioica*, *Matricaria chamomilla*, and *Murraya koenigii* on Rotenone-Exposed *D. melanogaster*: An attenuation of cellular, Biochemical, and Organismal Markers. ***Antioxidants*** **2022**	**7.675**
2.	Therapeutic Potential of Green-Engineered ZnO Nanoparticles on Rotenone-Exposed *D. melanogaster* (Oregon R+): Unveiling Ameliorated Biochemical, Cellular, and Behavioral Parameters. ***Antioxidants*** **2023**	**7.675**
3.	Proteinopathies: Deciphering Physiology and Mechanisms to Develop Effective Therapies for Neurodegenerative Disease. ***Molecular Neurobiology*** **2022**	**5.68**
4.	Synthesis of Green Engineered Silver Nanoparticles through *Urtica dioica*: An Inhibition of Microbes and Alleviation of Cellular and Organismal Toxicity in *Drosophila melanogaster*. ***Antibiotics*** **2022**	**5.222**
5.	Appraisal of the Antioxidant Activity, Polyphenolic Content, and Characterization of Selected Himalayan Herbs: Anti-Proliferative Potential in HepG2 Cells. ***Molecules*** **2022**	**4.927**
6.	Influence of the Gut Microbiota on the Development of Neurodegenerative Diseases. ***Mediators of Inflammation*** **2022**	**4.711**
7.	The evidence of *in-vivo* and *in-vitro* studies on microplastic and nano plastic toxicity in mammals. ***Physics and Chemistry of the Earth 2023***	**3.98**
8.	Protection of drug induced liver injuries through nutraceuticals via amelioration of Nrf2 signaling. ***Journal of the American Nutrition Association*** **2022**	**3.57**
9.	Protection of Phytoextracts against Rotenone-Induced Organismal Toxicities in *Drosophila melanogaster* via the Attenuation of ROS Generation. ***Applied Sciences*** **2022**	**2.679**
10.	Investigation of the Protective Effects of *Urtica dioica*, *Capsella bursa-pastoris* and *Inula racemosa* on Acetaminophen-Induced Nephrotoxicity in Swiss Albino Male Mice. ***Applied Sciences*** **2023**	**2.679**
11.	Book chapter: The aging: introduction, theories, principles and future prospective in Anti-Aging Drug Discovery on the Basis of Hallmarks of Aging **(Elsevier) 2022**.	-

APPENDIX I **LIST OF CONFERENCES**

S.NO.	CONFERENCES	DATE
1.	Participated in International Conference of Environmental, Agricultural, Chemical and Biological Sciences organized by Voice of Indian Concern for the Environmental **(VOICE 2021)** in association with Dept of Biotechnology, GLA University, Mathura, Uttar Pradesh, India and Dept of Zoology, Mercy College, Palakkad, Kerala, India.	24-26 January 2021
2.	Participated in International Conference on Sustainability: Life on Earth 2021 **(ICS-LOE 2021)** organized by School of Bioengineering and Biosciences, Lovely Professional University, Phagwara with Institute of Forest Productivity, Ranchi, India.	17-18 December 2021
3.	Participated in National Conference On Forensic Advancements & Technological Explorations 2022 **(FATE-2022)** organized by the Department of Forensic Science, Lovely Professional University, Phagwara with Patiala bureau of identification, Patiala, India.	25-26 November 2022
4.	Participated in 2nd International Conference on Plant Physiology and Biotechnology **(ICPPB-2023)** organized by the Division of Research and Development and Department of Molecular Biology and Genetic engineering, Lovely Professional University, In Association with Punjab State Council for Science and Technology.	20-21st April 2023

LIST OF INTELLECTUAL PROPERTY RIGHTS (IPR)

1. Therapeutic applications of phyto-nanotechnology for the treatment of neurodegeneration **(L-130386/2023)** May 2023**.**

2. Fabrication of green engineered zinc oxide nanoparticles and its therapeutic biomedical applications **(L-127809/2023)** May 2023.

LIST OF WORKSHOPS

1. **Training on The Care and Use of Laboratory Animals in Accordance with Ethical Guidelines** organized by School of Pharmaceutical Sciences, Human Resource Development Center (HRDC), Lovely Professional University (11-12 March 2022).

antioxidants

IF-7.675 MDPI

Article

Ethnopharmacological Effects of *Urtica dioica*, *Matricaria chamomilla*, and *Murraya koenigii* on Rotenone-Exposed *D. melanogaster*: An Attenuation of Cellular, Biochemical, and Organismal Markers

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Abstract: Natural antioxidants derived from plants have been proven to have significant inhibitory effects on the free radicals of living organisms during actively metabolization. Excessive production of free radicals increases the risk of neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and motor sclerosis. This study aimed to compare the ethnopharmacological effects of *Urtica dioica* (UD), *Matricaria chamomilla* (MC), and *Murraya koenigii* (MK) on the amelioration of rotenone-induced toxicity in wild-type *Drosophila melanogaster* (Oregon R^+) at biochemical, cellular, and behavioral levels. Phytoextracts were prepared from all three plants, i.e., UD, MC, and MK (aqueous and ethanolic fractions), and their bioactive compounds were evaluated using in vitro biochemical parameters (DPPH, ABTS, TPC, and TFC), UV-Vis, followed by FT-IR and HPLC. Third instar larvae and freshly eclosed flies were treated with 500 µM rotenone alone or in combination with UD, MC, and MK for 24 to 120 h. Following exposure, cytotoxicity (dye exclusion test), biochemical (protein estimation and acetylcholinesterase inhibition assays), and behavioral assays (climbing and jumping assays) were performed. Among all three plant extracts, MK exhibited the highest antioxidant properties due to the highest TPC, TFC, DPPH, and ABTS, followed by UD, then MC. The overall trend was MK > UD > MC. In this context, ethnopharmacological properties mimic the same effect in *Drosophila*, exhibiting significantly ($p < 0.05$) reduced cytotoxicity (trypan blue), improved biochemical parameters (proteotoxicity and AChE activity), and better behavioral parameters in the organisms cotreated with phyto extracts compared with rotenone. Conclusively, UV-Vis, FTIR, and HPLC analyses differentiated the plant extracts. The findings of this research may be beneficial in the use of select herbs as viable sources of phyto-ingredients that could be of interest in nutraceutical development and various clinical applications.

Keywords: antioxidants; acetylcholinesterase; 1,1-diphenyl-2-picrylhydrazyl; HPLC; medicinal plants; oxidative stress

Citation: Shabir, S.; Yousuf, S.; Singh, S.K.; Vamanu, E.; Singh, M.P. Ethnopharmacological Effects of *Urtica dioica*, *Matricaria chamomilla*, and *Murraya koenigii* on Rotenone-Exposed *D. melanogaster*: An Attenuation of Cellular, Biochemical, and Organismal Markers. *Antioxidants* **2022**, *11*, 1623. https://doi.org/10.3390/antiox11081623

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1. Introduction

The cellular redox status is determined by the balance between antioxidants and oxidants. The oxidative state of the cell is defined by an imbalance between these two, which can result in apoptosis or necrosis [1]. Reactive oxygen species (ROS) are primarily responsible for the high susceptibility of brain cells to oxidative stress [2]. Although oxygen is a relatively nonreactive substance, it can be metabolized in the body to create highly reactive free radicals, such as hydroxyl radicals (OH^-), superoxide anions (O_2^-), and many

antioxidants

IF-7.675 MDPI

Article

Therapeutic Potential of Green-Engineered ZnO Nanoparticles on Rotenone-Exposed *D. melanogaster* (Oregon R$^+$): Unveiling Ameliorated Biochemical, Cellular, and Behavioral Parameters

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Citation: Shabir, S.; Sehgal, A.; Dutta, J.; Devgon, I.; Singh, S.K.; Alsanie, W.F.; Alamri, A.S.; Alhomrani, M.; Alsharif, A.; Basalamah, M.A.M.; et al. Therapeutic Potential of Green-Engineered ZnO Nanoparticles on Rotenone-Exposed *D. melanogaster* (Oregon R$^+$): Unveiling Ameliorated Biochemical, Cellular, and Behavioral Parameters. *Antioxidants* **2023**, *12*, 1679. https://doi.org/10.3390/antiox12091679

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Abstract: Nanotechnology holds significant ameliorative potential against neurodegenerative diseases, as it can protect the therapeutic substance and allow for its sustained release. In this study, the reducing and capping agents of *Urtica dioica* (UD), *Matricaria chamomilla* (MC), and *Murraya koenigii* (MK) extracts were used to synthesize bio-mediated zinc oxide nanoparticles (ZnO-NPs) against bacteria (*Staphylococcus aureus* and *Escherichia coli*) and against rotenone-induced toxicities in *D. melanogaster* for the first time. Their optical and structural properties were analyzed via FT-IR, DLS, XRD, EDS, SEM, UV–Vis, and zeta potential. The antioxidant and antimicrobial properties of the fabricated ZnO-NPs were evaluated employing cell-free models (DPPH and ABTS) and the well diffusion method, respectively. Rotenone (500 µM) was administered to *Drosophila* third instar larvae and freshly emerged flies for 24–120 h, either alone or in combination with plant extracts (UD, MC, an MK) and their biogenic ZnO-NPs. A comparative study on the protective effects of synthesized NPs was undertaken against rotenone-induced neurotoxic, cytotoxic, and behavioral alterations using an acetylcholinesterase inhibition assay, dye exclusion test, and locomotor parameters. The findings revealed that among the plant-derived ZnO-NPs, MK-ZnO NPs exhibit strong antimicrobial and antioxidant activities, followed by UD-ZnO NPs and MC-ZnO NPs. In this regard, ethno-nano medicinal therapeutic uses mimic similar effects in *D. melanogaster* by suppressing oxidative stress by restoring biochemical parameters (AchE and proteotoxicity activity) and lower cellular toxicity. These findings suggest that green-engineered ZnO-NPs have the potential to significantly enhance outcomes, with the promise of effective therapies for neurodegeneration, and could be used as a great alternative for clinical development.

Keywords: zinc oxide nanoparticles; antioxidants; antibacterial agents; medicinal plants; green synthesis; oxidative stress

antibiotics

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Article

Synthesis of Green Engineered Silver Nanoparticles through *Urtica dioica*: An Inhibition of Microbes and Alleviation of Cellular and Organismal Toxicity in *Drosophila melanogaster*

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Abstract: Plant fractions have a diversity of biomolecules that can be used to make complicated reactions for the bioactive fabrication of metal nanoparticles (NPs), in addition to being beneficial as antioxidant medications or dietary supplements. The current study shows that *Urtica dioica* (UD) and biologically synthesized silver nanoparticles (AgNPs) of UD have antibacterial and antioxidant properties against bacteria (*Escherichia coli* and *Pseudomonas putida*) and *Drosophila melanogaster* (Oregon R+). According to their ability to scavenge free radicals, DPPH, ABTS, TFC, and TPC initially estimated the antioxidant potential of UD and UD AgNPs. The fabricated AgNPs were analyzed (UV–Vis, FTIR, EDS, and SEM) to determine the functional groups (alcohol, carboxylic acids, phenol, proteins, and aldehydes) and to observe the shape (agglomerated crystalline and rod-shaped structure). The disc diffusion method was used to test the antimicrobial properties of synthesized Ag-NPs against *E. coli* and *P. putida*. For 24 to 120 h, newly enclosed flies and third instar larvae of *Drosophila* were treated with UD and UD AgNPs. After exposure, tests for biochemical effects (acetylcholinesterase inhibition and protein estimation assays), cytotoxicity (dye exclusion), and behavioral effects (jumping and climbing assays) were conducted. The results showed that nanoparticles were found to have potent antimicrobial activity against all microbial strains tested at various concentrations. In this regard, ethno-medicinal characteristics exhibit a similar impact in *D. melanogaster*, showing ($p < 0.05$) significantly decreased cellular toxicity (trypan blue dye), enhanced biochemical markers (AChE efficacy and proteotoxicity), and improved behavioral patterns in the organism treated with UD AgNPs, especially in comparison to UD extract. The results of this study may help in the utilization of specific plants as reliable sources of natural antioxidants that may have been beneficial in the synthesis of metallic NPs, which aids in the production of nanomedicine and other therapeutic applications.

Keywords: antimicrobial activity; AgNPs; antioxidants; *Drosophila*; green synthesis; *Urtica dioica*

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Citation: Singh, M.P.; Shabir, S.; Deopa, A.S.; Raina, S.R.; Bantun, F.; Jalal, N.A.; Abdel-razik, N.E.; Jamous, Y.F.; Alhumaidi, M.S.; Altammar, K.A.; et al. Synthesis of Green Engineered Silver Nanoparticles through *Urtica dioica*: An Inhibition of Microbes and Alleviation of Cellular and Organismal Toxicity in *Drosophila melanogaster*. *Antibiotics* **2022**, *11*, 1690. https://doi.org/10.3390/antibiotics11121690

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Proteinopathies: Deciphering Physiology and Mechanisms to Develop Effective Therapies for Neurodegenerative Diseases

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Abstract

Neurodegenerative diseases (NDs) are a cluster of diseases marked by progressive neuronal loss, axonal transport blockage, mitochondrial dysfunction, oxidative stress, neuroinflammation, and aggregation of misfolded proteins. NDs are more prevalent beyond the age of 50, and their symptoms often include motor and cognitive impairment. Even though various proteins are involved in different NDs, the mechanisms of protein misfolding and aggregation are very similar. Recently, several studies have discovered that, like prions, these misfolded proteins have the inherent capability of translocation from one neuron to another, thus having far-reaching implications for understanding the processes involved in the onset and progression of NDs, as well as the development of innovative therapy and diagnostic options. These misfolded proteins can also influence the transcription of other proteins and form aggregates, tangles, plaques, and inclusion bodies, which then accumulate in the CNS, leading to neuronal dysfunction and neurodegeneration. This review demonstrates protein misfolding and aggregation in NDs, and similarities and differences between different protein aggregates have been discussed. Furthermore, we have also reviewed the disposal of protein aggregates, the various molecular machinery involved in the process, their regulation, and how these molecular mechanisms are targeted to build innovative therapeutic and diagnostic procedures. In addition, the landscape of various therapeutic interventions for targeting protein aggregation for the effective prevention or treatment of NDs has also been discussed.

Keywords Aggregates · Chaperone · Heat shock proteins · Misfolded protein · Neurodegenerative diseases

Introduction

One of the biggest challenges in the field of neuroscience is the disease-modifying treatment which include several neuroprotective and neurorestorative interventions that focus on slowing the progression of NDs. NDs are a plethora of debilitating and degenerative diseases, including Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD), frontotemporal dementia (FTD), traumatic encephalopathy, dementia with Lewy bodies, and prion diseases [1]. Despite having a difference in pathogenesis and clinical manifestation, these NDs share several common attributes, such as increased prevalence with age, progressive nature, selective neuronal loss, and synaptic dysfunction [2–6]. Importantly, the most prevalent and common feature of these NDs is the occurrence of misfolded proteins and their gradual accumulation, which is thought to be the main cause of these disorders [7–14]. Alpha-synuclein (α-Syn), amyloid-beta (Aβ), huntingtin (HTT), TAR DNA-binding protein 43 (TDP-43), Tau, and superoxide dismutase (SOD) are some of the proteins that are misfolded and accumulate in NDs [10–14]. In this review, we focused on Aβ, tau, α-Syn, HTT, and TDP-43, as they are the most common protein aggregates involved in the pathogenesis and progression of NDs. The distribution of the misfolded proteins involved in several diseases throughout the brain is depicted in Fig. 1, and Table 1 lists the different NDs caused by protein misfolding.

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molecules

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Article

Appraisal of the Antioxidant Activity, Polyphenolic Content, and Characterization of Selected Himalayan Herbs: Anti-Proliferative Potential in HepG2 Cells

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Abstract: Natural antioxidants derived from plants have played a vital role in preventing a wide range of human chronic conditions and provide novel bioactive leads for investigators in pharmacotherapy discovery. This work was designed to examine the ethnopharmacological role of *Urtica dioica (UD)*, *Capsella bursa-pastoris* (CBP), and *Inula racemosa* (IR). The total phenolic and flavonoid contents (TPC and TFC) were illustrated through colorimetric assays, while the antioxidant activity was investigated through DPPH and ABTS assays. The evaluation of phytochemicals by FT-IR of *UD* and CBP revealed high contents of aliphatic amines, while IR showed a major peak for ketones. The antioxidant activity, TPC and TFC were highest in the ethanol extract of UD, followed by CBP, and IR showed the lowest activity. All of the extracts revealed significant antioxidant capacities along a dosage gradient. Through a HPLC analysis at a wavelength of 280 nm, UD leaves demonstrated an intense peak of quercetin, and the peak for rutin was less intense. CBP (whole plant), instead, demonstrated a major yield of rutin, and a peak for quercetin was not observed in CBP. IR (rhizomes) showed both quercetin and rutin. All of the extracts were significantly cytotoxic to HepG2 cells after 48 h with the trend IR > UD > CBP. The outcomes of this study may be effective in the selection of specific plants as realistic sources of the bioactive components that might be useful in the nutraceutical progression and other biomedical efficacies.

Keywords: antioxidant activity; Himalayan herbs; HPLC; FT-IR; HepG2; anti-proliferative activity; oxidative stress

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Review Article

Influence of the Gut Microbiota on the Development of Neurodegenerative Diseases

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Neurodegenerative disorders are marked by neuronal death over time, causing a variety of cognitive and motor dysfunctions. Protein misfolding, neuroinflammation, and mitochondrial and protein clearance system dysfunction have all been identified as common pathways leading to neurodegeneration in recent decades. An altered microbiome of the gut, which is considered to play a central role in diseases as well as health, has recently been identified as another potential feature seen in neurodegenerative disorders. An array of microbial molecules that are released in the digestive tract may mediate gut-brain connections and permeate many organ systems, including the nervous system. Furthermore, recent findings from clinical as well as preclinical trials suggest that the microbiota of the gut plays a critical part in gut-brain interplay and that a misbalance in the composition of the gut microbiome may be linked to the etiology of neurological disorders (majorly neurodegenerative health problems); the underlying mechanism of which is still unknown. The review aims to consider the association between the microbiota of the gut and neurodegenerative disorders, as well as to add to our understanding of the significance of the gut microbiome in neurodegeneration and the mechanisms that underlie it. Knowing the mechanisms behind the gut microbiome's role and abundance will provide us with new insights that could lead to novel therapeutic strategies.

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The evidence of *in-vivo* and *in-vitro* studies on microplastic and nano plastic toxicity in mammals: A possible threat for an upcoming generation?

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ABSTRACT

Plastic pollution is a pressing global environmental concern, exerting significant impacts on both aquatic and terrestrial ecosystems in recent decades. The detrimental effects of plastic on various species within these habitats have raised serious alarm. Regrettably, human exposure to microplastics (MPs) and nanoplastics (NPs) through contaminated food has become unavoidable, but the comprehensive extent of their consequences remains largely unexplored. This review aims to advance existing research and conduct a comprehensive evaluation of current studies pertaining to the potential toxicity and negative impacts of micro- and nano-plastics on soil, water, and land, with a specific focus on their implications for human health. Through meticulous examination of the existing literature, we aim to provide a succinct overview of the potential risks associated with micro and nano plastics, thereby guiding and emphasizing the direction for future research in this domain and addressing critical knowledge gaps. The study underscores that extant research has established a clear association between micro and nano plastics and their adverse effects on the digestive system, cell lines, and reproductive health. Nevertheless, deeper investigations are imperative to achieve a better understanding of the cellular, molecular, neurological, and systemic toxicity caused by these minute plastic particles. Information presented in the review provides a scientific understanding of the hazards posed by micro and nano plastics to both the environment and human health is essential for formulating effective mitigation strategies and regulatory measures.

1. Introduction

Plastic pollution in the environment is receiving a lot of attention currently and inappropriate unloading of discarded or abandoned plastic waste pollutes the environment (Pinto et al., 2019). Since the 1940s, when the widespread use of plastics in fields as diverse as industry, agriculture, medicine, and consumer goods emerged, the plastics sector has grown exponentially (Peng et al., 2022). It's not unexpected that people are being exposed to microplastics (anything less than 5 mm in size) since they are pervasive environmental pollutants found in water and food (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2016). Secondary microplastics, such as those released from strewn plastic trash, are the consequence of the breakdown of primary microplastics, which are manufactured purposely for technical uses such as abrasive in personal care items (Andrady, 2017). The removal of household wastewater discharge, sewage ooze garbage, and plastic waste from agrarian soils is a major challenge for natural decontamination (Chae and An, 2018). Plastic is widely utilized due to its exceptional utility in facilitating the convenient transportation of daily essential items for individuals. Its practicality stems from its versatility, durability, and lightweight properties, which make it an ideal material for various applications in modern life. (Mafuta et al., 2021). Once in the

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REVIEW

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Protection Against Drug-Induced Liver Injuries Through Nutraceuticals via Amelioration of Nrf-2 Signaling

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ABSTRACT

Hepatotoxicity caused by the overdose of various medications is a leading cause of drug-induced liver injury. Overdose of drugs causes hepatocellular necrosis. Nutraceuticals are reported to prevent drug-induced liver failure. The present article aims to review the protection provided by various medicinal plants against hepatotoxic drugs. Ayurveda is considered a conventional restorative arrangement in India. It is consistently used for ages and is still used today to cure drug-induced hepatotoxicity by focusing on antioxidant stress response pathways such as the nuclear factor erythroid-2 (Nrf-2) antioxidant response element signaling pathway. Nrf-2 is a key transcription factor that entangles Kelch-like ECH-associating protein 1, a protein found in the cell cytoplasm. Some antioxidant enzymes, such as gamma glycine cysteine ligase (γ-GCL) and heme oxygenase-1 (HO-1), are expressed in Nrf-2 targeted genes. Their expression, in turn, decreases the stimulation of hepatic macrophages and induces the messenger RNA (mRNA) articulation of proinflammatory factors including tumor necrosis factor α. This review will cover various medicinal plants from a mechanistic view and how they stimulate and interact with Nrf-2, the master regulator of the antioxidant response to counterbalance oxidative stress. Interestingly, therapeutic plants have become popular in the medical sector due to safer yet effective supplementation for the prevention and treatment of new human diseases. The contemporary study is expected to collect information on a variety of therapeutic traditional herbs that have been studied in the context of drug-induced liver toxicity, as nutraceuticals are the most effective treatments for oxidative stress-induced hepatotoxicity. They are less genotoxic, have a lower cost, and are readily available. Together, nutraceuticals exert protective effects against drug-induced hepatotoxicity through the inhibition of oxidative stress, inflammation, and apoptosis. Its mechanism(s) are considered to be associated with the γ-GCL/HO-1 and Nrf-2 signaling pathways.

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KEYWORDS
Liver injuries; nutraceuticals; Nrf-2 signaling; acetaminophen; γ-GCL; HO-1; chemoprevention

KEY TEACHING POINTS

- The liver is the most significant vital organ that carries out metabolic activities of the body such as the synthesis of glycogen, the formation of triglycerides and cholesterol, as well as the formation of bile.
- Acute liver failure is caused by the consumption of certain drugs; drug-induced liver injury is the major condition.
- The chemopreventive activity of nutraceuticals may be related to oxidative stress reduction and attenuation of biosynthetic processes involved in hepatic injury via amelioration of the nuclear factor erythroid-2 (Nrf-2) signaling pathway.
- Nrf-2 is a key transcription factor that is found in the cell cytoplasm resulting in the expression of various genes such as gamma glycine cysteine ligase and heme oxygenase-1.
- Nutraceutical-rich phytochemicals possess high antioxidant activity, which helps in the prevention of hepatic injury.

Abbreviations: γ-GCL =: gamma glycine cysteine ligase; ALF: acute liver failure; ALP: serum alkaline phosphatase; ALT: alanine aminotransferase; APAP: acetaminophen; APCs: antigen-presenting cells; AST: aspartate aminotransferase; BTB: broad complex; CYP: cytochrome P450; DILI =: drug-induced liver injury; FDA: US Food and Drug Administration; GCL: glutamate-cysteine ligase; GCLC: glutamate cysteine ligase catalytic unit; GCLM: glutamate cysteine ligase modifier; GI: gastrointestinal; GSH: glutathione; HO-1 =: heme oxygenase-1; IFN: interferon, IL=interleukin; INH: isoniazid; KCs: Kupffer cells; KEAP1: Kelch-like ECH-associating protein 1; LDH =: lactate dehydrogenase; LPO =: lipid peroxidation; MAPK =: mitogen-activated protein kinases; mRNA: messenger RNA; MsrA: methionine sulfoxide reductase A; NAC =: N-acetyl-L-cysteine; NK: natural killer; NO: nitric oxide; Nrf-2 =: nuclear factor erythroid-2, ONOO=peroxynitrite; RNS: reactive nitrogen species; ROS =: reactive oxygen species; SOD =: superoxide dismutase; TM: tissue macrophages

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Article

Protection of Phytoextracts against Rotenone-Induced Organismal Toxicities in *Drosophila melanogaster* via the Attenuation of ROS Generation

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Abstract: Nutraceuticals play an essential role in the reduction in free radical generation in cells. A similar idea was used in the present study to determine the effects of aqueous extracts on the organismal toxicities in a nontarget organism, *Drosophila melanogaster*, known as the fruit fly. *Punica granatum* (peel and pulp), *Carica papaya* (peel), *Foeniculum vulgare* (seeds), *Trigonella foenum-graecum* (seeds), and *Urtica dioica* (leaves) extracts were employed in this study. The organismal or behavioral effects in rotenone-, and rotenone- and phytoextract-treated flies were evaluated using wild-type *Drosophila melanogaster* (Oregon R$^+$). Reactive oxygen species (ROS) and behavioral parameters (climbing ability, memory power, emergence, and reproductive potential) were investigated. *Urtica dioica* leaves, *Punica granatum* peel, and pulp elicited maximal amelioration in *Drosophila*, although not at the same intensity, and all exhibited a varied degree of improvement in different assays. Most extracts with their potent active components (phenols, tannins, flavonoids, and amino acids) revealed a protective action against rotenone-induced toxicities at the organismal level in the stated parameters above. Interestingly, different strains and parameters had varied improvement tendencies. Thus, *Drosophila* may be used as a suitable in vivo animal model for such investigations, and the usage of phytoextracts may prevent a variety of disorders, including neurodegeneration. The results of this study may help in the use of specific herbs as reliable sources of phytoingredients that may be useful in developing nutraceuticals and in other clinical uses.

Keywords: phytoextracts; organismal parameters; free radicals; neurodegeneration; oxidative stress

1. Introduction

Free radical damage to lipids, polypeptides, and DNA, and its impact on apoptosis, cell proliferation, and ion transport contribute to diseases including neurodegenerative disorders. The loss of dopaminergic neurons in the substantia nigra is a hallmark of

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Article

Investigation of the Protective Effects of *Urtica dioica*, *Capsella bursa-pastoris* and *Inula racemosa* on Acetaminophen-Induced Nephrotoxicity in Swiss Albino Male Mice

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Abstract: Acetaminophen (APAP) is the most commonly used nonprescription antipyretic-analgesic drug. This medication is thought to be safe at the suggested dosage (4 g/24 h), but its overdose (up to 2.5 g/kg) can cause severe injuries to the human body, including renal injury. APAP has various toxic effects on nephrons, as it leads to an excessive free radical generation that, in turn, results in a disturbance in the redox homeostasis of cells, causing oxidative stress. To replenish this oxidative stress, there is an ultimate urge for natural therapies that can retain the cellular homeostasis of nephrons by diminishing the overdose impression of acetaminophen. The principle objective of this work is to appraise nephrotoxicity due to APAP and its amelioration through the antioxidant properties of aqueous extracts of selected medicinal plants: *Urtica dioica*, *Capsella bursa-pastoris*, and *Inula racemosa* (UD, CBP, and IR, respectively). The pH stability of the nutraceuticals used was examined by determining the impact of pH 4, pH 7 and pH 9 on the DPPH radical scavenging activity of aqueous plant extracts. Gas chromatography-Mass spectroscopy (GC–MS) analytical technique was performed to determine the volatile organic phytochemical profiles of all three medicinal plants. Male Swiss albino mice were used for the present investigation. The animals were distributed into five groups of (n = 6), a total of 30 mice, for in vivo analysis. Group 1 served as the control group; group 2 received a single IP dose of APAP (600 mg/kg); group 3 received APAP pretreated with UD (300 mg/kg); group 4 received APAP pretreated with CBP (300 mg/kg); and group 5 received APAP pretreated with IR (300 mg/kg). Overdose of the APAP- induced a significant ($p < 0.05$) alterations in the total protein concentration, weight and the nephrological architecture in renal tissue, as observed through biochemical assays and histopathological examinations. Due to nephrotoxicity, there was a substantial ($p < 0.05$) drop in body weight and total protein contents in the APAP alone group when compared to the treatment groups. There was remarkable protection against APAP-induced alterations in the total protein of renal homogenate in the treatment groups. Histopathological analysis (H&E staining) of the mice kidneys indicated severe deterioration in the APAP alone group, whereas the therapy groups showed considerable nephroprotection towards APAP-induced abnormalities. The biochemical findings and histopathological study of the kidneys revealed that the herbal extracts (UD, CBP, and IR) have a nephroprotective potential against APAP-induced nephropathy. The trend

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Book Chapter

Anti-Aging Drug Discovery on the Basis of Hallmarks of Aging
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Chapter 1 - The aging: introduction, theories, principles, and future prospective

Shabnam Shabir, Mahendra P. Singh

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Abstract

Aging is psychosocially and biologically defined as being older. An aged or geriatric patient is defined as a person whose biological age is advanced. Aging can be characterized as a deterioration of the physiological functions essential for survival and fertility that is time-related. Aging is a complex spatial and temporal hierarchy of dynamic activities that are integrated over the life cycle of a complex dance. Thus aging is not easily dissected into disjoint subprocesses; it is a dynamic, multidimensional, hierarchical process. In most body structures, aging is followed by incremental modifications. Aging biology research focuses on understanding both the cellular and molecular mechanisms that underlie these changes and those that accompany the onset of age-related diseases. As time passes, aging takes place in a cell, an organ, or the whole body. It is a phase of every living thing that goes on through the whole adult life cycle. Aging is an organism's sequential or incremental transition that results in an increased risk of fatigue, illness, and death. Aging is multifaceted. Therefore, there are various hypotheses each of which may clarify one or more aspects of aging. Aging in humans reflects the accumulation over time of changes in a human being that may involve social, psychological, and physical changes. Recent hypotheses are assigned to the concept of damage, whereby the accumulation of damage (including DNA oxidation) may cause biological systems to fail, or to the concept of programmed aging, whereby problems with internal processes (epigenomic maintenance can cause aging such as DNA methylation) are correlated with the causes of aging.

Previous Next

Keywords

Aging; senescence; aging theories; antiaging therapies; stem cell therapy

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