Investigating the therapeutic potential of *Rosmarinus Offficinalis* and *Bacillus Clausii* in MPTP-induced mouse model of Parkinson's Disease

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Investigating the therapeutic potential of *Rosmarinus Offficinalis* and *Bacillus Clausii* in MPTP-induced mouse model of Parkinson's Disease

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Healthcare Biotechnology

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This thesis is dedicated to my parents for their endless love and support

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ABBREVIATIONS

PD	**Parkinson's Disease**
MPTP	**1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine**
SOD	**Superoxide Dismutase**
IL-6	**Interleukin-6**
TNF-α	**Tumor Necrosis Factor-alpha**
SNCA	**Alpha synuclein**
APOE	**Apolipoprotein-E**
PRKN	**Parkin**
ADME	**Absorption Distribution Metabolism and Excretion**
SNpc	**Substantia nigra pars compacta**
ROS	**Reactive Oxyegen Species**
CFU	**Colony Formulation Unit**
µl	**Microliter**
PDD	**Parkinson Disease Dimentia**
REM	**Rapid Eye Movement**
DAT	**Dopamine transporter**
SNr	**Substantia nigra reticulata**
GPi	**Globus Pallidus Internus**
NO	**Nitric Oxide**

AADC	**Aromatic L-Aminoacid Decarboxylase**
MAO-B	**Monoamine Oxide-B**
DBS	**Deep Brain Stimulation**
CBT	**Cognitive Behavior Therapy**
GBA	**Gut-Brain Axis**
HPA	**Hypothalamic-Pituitary Adrenal**
BBB	**Blood Brain Brain**
NDD	**Neurodegenerative Disorder**
rpm	**Revolution Per Minute**
UA	**Ursolic Acid**
CA	**Carnosic Acid**
RA	**Rosmarinic Acid**

Abstract

Parkinson's disease (PD) is a complex neurodegenerative disease affecting 1% of people age 60 and above worldwide. The disease is characterized by the ever-evolving loss of dopaminergic neurons in the substantia nigra pars compacta, alpha synuclein aggregation, oxidative stress, and neuroinflammation, all of which contributes to motor dysfunction, including postural instability, rigidity, bradykinesia, and tremors. Despite advancements in PD management, current treatments remain limited to symptomatic srelief and fail to target underlying disease mechanisms to halt progression. Therefore, the present study investigated the neuroprotective effects of *Rosmarinus officinalis* extract and *Bacillus clausii*, focusing on motor coordination, neuroinflammation, and oxidative stress in a 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP)-induced PD mouse model.

MPTP (30 mg/kg, intraperitoneally) was used to develop PD mouse model. Mice were divided into eight groups (n=8), where the four groups were given MPTP intraperitoneally followed by treatment with *R.officinalis* (120 mg/kg, intraperitoneally) extract, *Bacillus clausii* ($2x10^9$ CFU, oral) and a combination treatment of both. The other four groups received *R. officinalis* extract, *Bacillus clausii* and saline only. Behavioral tests were performed including open field test, pole test and cylinder test to asess bradykinesia, locomotor activity, motor disabilities and forelimb asymmetry, respectively. The open field test uncovered expanded exploratory activity in treatment groups, particularly in the combination treatment group ($p<0.0001$). Significant improvement ($p<0.0001$) in the movements in the combination treatment group was observed through pole test. Similarly, motor balance and forelimb symmetry was also restored in the treatment groups, with the combination treatment groups

achieving the most significant results ($p<0.001$), assessed via the cylinder test. Histopathological examination using Hematoxylin & Eosin (H&E) staining showed marked preservation of neuronal density in the substantia nigra and hippocampus in the combination treatment group compared to the MPTP-treated group. Gene expression analysis revealed significant amelioration of antioxidants (SOD1, SOD2) and pro-inflammatory cytokines (IL-6, TNF-α) in the combination treatment group ($p<0.0001$) in comparision to MPTP-treated groups, supporting its antioxidative and anti-inflammatory effects. Molecular docking analyses of bioactive compounds of *R. officinalis*, including carnosic acid (CA) and ursolic acid (UA), demonstrated strong binding affinities to key PD-associated proteins, including SNCA, PRKN, APOE, SOD1, IL-6, SOD2, and TNF-α, indicating their potential role in modulating oxidative stress and inflammation pathways. The analysis of pharmacokinetic properties and drug likeness through (Absorption, Distribution, Metabolism, and Excretion) ADME and Lipinski filter analysis revealed the drug-relevant features of the compounds affirming their promising potential as therapeutic candidates for PD.

The results demonstrate the neuroprotective potential of *R.officinalis* and *B.clausii* through their antioxidative, anti-inflammatory, and neuroprotective properties. These analysis gives foundation to additional investigation of their mechanistic roles and translational application as synergistic treatments to mitigate PD onset and progression.

Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder that affects the nervous system and particularly the functions associated with movement. This can be credited to the persistent loss of brain cells known as dopaminergic neurons, situated in the substantia nigra. This results in loss of level of dopamine (DA) in brain which ultimately causes many pathological features that are muscle rigidity, postural instability, slowness of movemnets etc (Lozano et al., 2019). A definitive cause for PD is yet to be laid out. By the by, it is broadly acknowledged that various elements including hereditary, ecological, and age-related ones are associated with the advancement of this problem (Kalia & Lang, 2016). In the mechanism of neurodegeneration associated with PD, oxidative stress and neuroinflammation are considered major contributors (Hirsch & Hunot, 2009).

PD has a varying occurrence worldwide, with around 6.1 million individuals affected globally as of 2016. The frequency and occurrence of PD rise with advancing age, rendering it mostly a condition that affects older individuals. The prevalence of this condition in those aged 60 and above varies between 1% and 2%, and increases to 3% to 5% in those aged 85 and above (Pringsheim et al., 2014). Men have a higher likelihood of developing PD compared to women, with a male-to-female ratio of roughly 1.5:1. Because of the rising number of older individuals, it is viewed as that the patients of PD will increment twofold by 2040. This will provide substantial difficulties to public health and the economy (Dorsey et al., 2018a). The disease not only affects the individuals but also creates a financial burden on careers and healthcare systems.

Around 10% to 15% of PD cases are of genetic origins. Out of these mutations, the most well-known are those located within the LRRK2, SNCA, PARK2, PARK7, PINK1 genes. Brown et al. has shown that one of the environmental variables which

holds the probability of PD is the utilization of pesticides, metals, and solvents (Brown et al. 2005). The etiopathology of PD is multi-layered and perplexing (Dorsey et al., 2018a). Neurodegenerative changes happening in the substantia nigra is the primary cause of the illness with a prevalent decrease of dopamine. This outcomes in lack of dopamine in the striatum which upsets the ordinary action of the circuits in the basal ganglia (Obeso et al., 2000). The lack of dopamine significantly affects both the immediate and aberrant pathways of the basal ganglia which results in many motor symptoms of PD (Albin et al., 1989).

The second significant phase of PD is portrayed by the presence of intracellular incorporations named as Lewy bodies: these are principally comprised of protein α-synuclein (Spillantini et al., 1997). Though the precise mechanisms through which aggregates of α-synuclein become neurotoxic remain not completely unveiled, it is assumed that most of them relate to imbalance of cellular homeostasis, malfunction of mitochondria and disturbed pathways of protein degradation (Wong & Krainc, 2017).

The dysfunction of the mitochondria can go probably as a driving part in the development of the PD. Mitochondria have an essential impact in the energy production of the cells, and any dysregulation in their cycle causes oxidative stress hence achieving damage to the nerve cells (Exner et al., 2012). Positive mutations in familial qualities connected with the appropriate working of mitochondria, like PARKIN and PINK1, have been related with familial types of PD (Klein & Westenberger, 2012).

Neuroinflammation is known to be a significant factor in PD pathogenesis and is related with the supported actuation of occupant immune defense cells in the mind, known as microglia. At the point when microglia are actuated they discharge provocative cytokines and arbiters like interleukin-6 (IL-6) and tumor necrosis factor (TNF-α). It is believed that prolonged inflammation of the brain may increase the rates of

neurodegeneration and therefore it may be worth considering for therapeutic approaches (Hirsch & Hunot, 2009).

Current treatments of PD have focused on natural treatment of PD. *Rosmarinus officinalis* commonly known as rosemary, is a traditional medicine which is known for its multiple pharmacological effects. It is rich in bioactive compounds like carnosol, carnosic acid (CA), and rosmarinic acid (RA) etc. known to as potent antioxidants and anti-inflammatory agents (Hirsch & Hunot, 2009).

Research on neurodegenerative disorders, for example, PD has brought the gut-brain axis to the very front of interest (Mulak & Bonaz, 2015). The gut-microbiome can likewise influence the capability of the brain by various pathways, like the regulation of systemic inflammation and oxidative stress (Sampson et al., 2016). Changes in gut microbiota has been seen in patients with PD, suggesting the chance of keeping up with the strength of the gut and that of the sensory system in general (Tursi et al., 2013). On account of PD, *B. clausii* gives a security against the illness by reengineering the gut microbiota and controlling the degrees of inflammation in the body.

Oxidative stress is caused when the reactive oxygen species (ROS) exceeds the removal or damage control mechanisms from the brain. Physical and pharmacological examinations show that PD can be considered as particular weakness of dopaminergic neurons in the substantia nigra because of their elevated metabolism, dopamine autoxidation, which expands the degrees of oxidative pressure (ROS) (Prendecki et al., 2020). Without host antioxidant defenses, particularly superoxide dismutase and catalase, oxidative damage is enhanced. Hence, causing further damage and death of the neurons (Beal et al., 2003).

The goal of the present study is to explore mouse models of parkinsonism, the neuroprotective effects of *R. officinalis* and *B. clausii* with respect to oxidative stress

and neuroinflammation in detail. We will focus this discussion mainly on important genes that are known to be associated with these pathways such as superoxide dismutase SOD-1, SOD-2, IL-6, and TNF-α (Kim et al., 2018). The assessment of motor functions and potential therapeutic advantages will be conducted through the use of the open field test, pole test, and cylinder test (Sedelis et al., 2001). Likewise, a histological examination will be led using hematoxylin and eosin (H&E) stains to survey neuronal density, specifically to search for indications of neurodegeneration (Miller et al., 2013). Moreover, docking anaysis will likewise be utilized to assess the collaboration of dynamic constituents of R. *officinalis* alongside the important neuro-aggravation and oxidative pressure related proteins (Hirsch et al., 2016).

The computational research will provide vital insights into the molecular mechanisms that explain the neuroprotective advantages of *R.officinalis* and *Bacillus clausii*. The primary objective is to create novel therapeutic strategies that precisely address oxidative stress and neuroinflammation.

1.1 Aims and Objectives

The study aimed to investigate the potential of *R. officinalis* and *Bacillus Clausii* in a mouse model of PD.

- To evaluate the neuroprotective effects of *R. officinalis* and *B. clausii* through behavioral analysis.
- To investigate the neuroprotective properties of *R. officinalis* and *B. clausii* on the structural integrity of neurons in the hippocampus and substantia nigra.
- To evaluate the effects of *R.officinalis* and *B.clausii* on oxidative stress and neuroinflammation associated genes.
- To assess the interaction potential of bioactive compounds of *R. officinalis* with proteins implicated in PD through in silico methods.

Literature Review

2.1 Parkinson's Disease

PD is a chronic neurodegenerative problem that influences the motor system and is described by the loss of dopaminergic nerve cells. The problem is described by the progressive loss of dopaminergic neurons in the substantia nigra pars compacta, which is responsible for producing dopamine. The decrease in the levels of dopamine results in a number of motor and non-motor symptoms of PD which includes resting tremors, bradykinesis, muscle rigidity etc (Kalia & Lang, 2015). PD , initially documented by Dr. James Parkinson in 1817 in his publication "An Essay on the Shaking Palsy," is now acknowledged as a widespread and incapacitating neurological ailment that impacts millions of individuals globally.

Neuronal degeneration in the substantia nigra, bringing about an absence of dopamine in the striatum, and the presence of strange collections of α-synuclein inside cells are the trademark clinical highlights of PD. While the clinical finding of PD is basically founded on the perception of bradykinesia and other motor symptoms, it is critical to take note that the condition is likewise joined by numerous non-motor symptoms also that add to the absolute degree of debilitation (Poewe et al., 2017).

2.2 Prevalence and impact

PD is the second most predominant neurological problem, after Alzheimer's sickness. It influences around 1% of those matured 60 or more (Pringsheim et al., 2014). Men have a higher likelihood, around 1.5 times, of developing PD compared to women. However, the specific causes for this gender gap are yet unknown. Geographic disparities uncover more prominent event rates in industrialized districts like North America and Europe as opposed to Asia and Africa, with North America recording

about 329 cases for every 100,000 people contrasted with around 114 cases for each 100,000 in Asia (Marras et al., 2018). These variations might be impacted by genetic predisposition, environmental factors, and healthcare protocols. Ethnic and racial differences suggest that those of Caucasian descent have a greater risk in comparison to individuals of African American and Asian descent. Primary risk factors for PD incorporate propelling age, male sex, family hereditary variations (like SNCA, PARK2, LRRK2, PINK1, and DJ-1), openness to natural contaminations such pesticides and metals, and a past filled with head injury. On the other hand, cigarette smoking and caffeine utilization appear to bring down the possibility of developing PD. Because of the worldwide populace maturing, it is assessed that the predominance of PD will twofold by 2040. This features the dire requirement for progressing examination into the causes, anticipation, and treatment of PD, as well as the improvement of general wellbeing methodologies to address the rise of this neurodegenerative condition (Dorsey et al., 2018b).

PD has a significant effect, influencing the people with it, yet additionally their families and the medical care framework. PD fundamentally appears with engine side effects like quakes, bradykinesia (decreased speed of development), inflexibility, and postural precariousness. Engine weaknesses lead to huge handicap, diminishing the general personal satisfaction and expanding the requirement for help with everyday exercises. Moreover, PD is related with other non-motor syptoms, including mental disability, state of mind issues (like significant misery and tension), aggravations in rest designs, breakdown of the autonomic sensory system, and anomalies in tactile discernment. The non-motor syptoms can be similarly pretty much as weakening as the engine side effects, subsequently expanding the trouble of dealing with the condition (Hirsch et al., 2016).

The increasing prevalence of PD, driven by a globally ageing population, emphasizes the urgent need for effective treatments and comprehensive care strategies. In addition, PD has a significant social impact, leading to emotional and psychological impact on both patients and their careers (Ascherio & Schwarzschild, 2016).

2.3 Clinical manifestation and diagnosis

PD is characterized by diverse motor and non-motor symptoms that progressively emerge, reflecting the steady decline of neurons in the brain responsible for generating dopamine. To establish the diagnosis of PD, other disorders that exhibit comparable symptoms are systematically eliminated (Jankovic et al., 2008).

2.3.1 Motor Symptoms

Motor symptoms refer to physical manifestations or abnormalities that affect movement or coordination. The vitally engine side effects of PD are quake, bradykinesia (diminished development speed), solidness, and postural unsteadiness. These symptoms usually appear unevenly and advance gradually over a span of several years (Weintraub et al., 2008). The symptoms include:

Resting Tremor: It refers to a regular shaking movement that often happens when the body is at rest.

Bradykinesia: It is a condition marked by a widespread decrease in the speed of intentional movement, impacting tasks like fastening garments, walking, and making facial expressions. Motor functions may exhibit diminished amplitude and decreased velocity, resulting in a decline in face mobility (reduced frequency of blinking and limited range of facial expressions) (Schapira et al., 2017).

Rigidity: Rigidity frequently presents as muscular inflexibility in the extremities, neck, or torso, resulting in challenges in initiating and carrying out movements (Postuma et al., 2015).

Postural instability: It refers to a decline in balance and coordination, which becomes more noticeable as the condition progresses. Patients may experience challenges in initiating movement, executing turns, and keeping a stable posture, which can elevate the likelihood of falls.

2.3.2 Non-Motor Symptoms:

Aside from the actual impediments, PD is connected to numerous non-motor syptoms that significantly affect the singular's personal satisfaction:

Autonomic Dysfunction: It is characterized by symptoms such as constipation, urine urgency, nocturia, and orthostatic hypotension, which can prompt unsteadiness and falls because of a diminishing in circulatory strain after standing (Schapira et al., 2017).

Cognitive impairment: It is prevalent in individuals with PD, particularly in the form of mild cognitive impairment. This condition primarily impacts cognitive abilities such as attention, executive function, and memory. PD dementia (PDD) is a condition where cognitive deterioration can advance to the point of dementia (Ascherio & Schwarzschild, 2016).

Mood disorders: like wretchedness and tension, are regularly tracked down in individuals with PD, ordinarily happening before the presence of mototr symptoms. Emotional disturbances can lead to significant functional limitations and may require specific treatment strategies.

Sleep disorders: PD is related with many rest issues, including a sleeping disorder, fretful legs condition, and rapid eye movement (REM) rest conduct jumble (Weintraub et al., 2008).

Sensory Symptoms: Hyposmia, a condition portrayed by a diminished capacity to identify smells, is usually found in individuals with PD and may start before the beginning of engine side effects by numerous years. Moreover, patients might encounter extra tactile anomalies, like vision hindrances and agony challenges.

2.3.3 Diagnosis

Diagnosing PD can pose challenges, particularly during the initial phases when symptoms may be subtle or not directly linked to the condition. The diagnosis is often determined using clinical criteria and may need many sequential steps:

Medical History and Physical Examination: A thorough assessment that involves a careful investigation into the onset and progression of symptoms, familial medical history, and potential exposure to risk factors. The physical examination assesses motor function, gait, posture, tremor characteristics, and the reaction to levodopa, a dopamine precursor (Postuma et al., 2015).

Neuroimaging methods: such as dopamine transporter (DAT) scans or structural MRI, may be employed to validate clinical observations and eliminate other causes of Parkinsonism, but they are not essential for diagnosis (Jankovic et al., 2008).

Differential Diagnosis: PD should be separated from other neurodegenerative problems, for example, various framework decay and moderate supranuclear paralysis, as well as medication prompted Parkinsonism, fundamental quake, and vascular parkinsonism (Poewe et al., 2017).

2.4 Pathophysiology and Neurodegeneration

2.4.1 Dopamine deficiency and basal ganglia dysfunction

The dopaminergic system assumes a crucial part in the pathogenesis of PD. The brain organization, contained neurons that pass signals on through the synapse dopamine, assumes a fundamental part in co-ordinating development and a few mental processes.The essential obsessive qualities of PD remember the deficiency of pigmentation for the substantia nigra pars compacta (SNpc) and the degeneration of dopaminergic neurons as shown in figure 2.1. The deficiency upsets the harmony between the immediate and aberrant pathways of the basal ganglia, prompting disabled motor performance (Chakravarty et al., 2022).The direct course advances development by working with the actuation of the thalamocortical framework through thalamic disinhibition. As an outcome, there is an ascent in cortical motor output , prompting an expansion of development (Sanders et al., 1996). The indirect course hinders development by expanding thalamocortical hindrance through expanded action in the substantia nigra pars reticulata (SNr), and globus pallidus internus (GPi) prompting decreased cortical motor yield (Sanders et al., 1996).

2.4.2 Lewy bodies and α-Synuclein Aggregation

Lewy bodies are fundamental in the development of PD. The essential constituents of the lewy body comprises of α-synuclein and ubiquitin. α-synuclein is the essential protein found in Lewy bodies (Fares et al., 2014). PD is portrayed by the strange collapsing and collection of proteins, prompting the development of insoluble protein bunches in neuron (Hirsch et al., 2016).

2.4.3 Neuroinflammation

Neuroinflammation assumes a significant part in the advancement of PD. It is portrayed by the improvement of strange protein accumulation in particular α-Synuclein, which cause the enactment of microglia because of the production of Lewy bodies inside neurons (Kohutnicka et al., 1998). Microglia that have been activated secrete pro-inflammatory cytokines. These inflammatory chemicals can cause further harm to neurons by triggering localized inflammation. Neuroinflammation is related with the creation of reactive oxygen species (ROS) and other unsafe synthetic compounds (Hirsch & Hunot, 2009).To develop effective therapies for PD and improve patient outcomes, it is crucial to get a thorough understanding of neuroinflammation and particularly target it as a therapeutic approach.

2.4.4 Oxidative stress

Oxidative stress assumes a urgent part in the decay of dopaminergic neurons in PD. Oxidative Stress is the condition when there is an inconsistent measure of ROS.The unreasonable creation of ROS in the brain might make sense of the significant impact that these reactive species have on PD. The brain consumes around 20% of the body's oxygen supply, a huge piece of which is changed over into (ROS (Prendecki et al., 2020). Moreover, critical degrees of nitric oxide (NO) are available in the mind because of the presence of nitric oxide synthase (NOS). The presence of ROS is an essential component adding to the demise of dopaminergic neurons in the minds of people with PD. The development of these ROS is brought about by the breakdown of dopamine, decreased degrees of glutathione (GSH), and expanded degrees of iron and calcium in the substantia nigra pars compacta (SNpc). Moreover, the mind contains significant amounts of polyunsaturated unsaturated fats. At the point when these unsaturated fats

are exposed to oxidative pressure, they go through lipid peroxidation, bringing about the age of adverse synthetic compounds (Dias et al., 2013).

2.4.5 Mitochondrial dysfunction

Mitochondria, the organelles inside cells, are powerhouse for the development of energy. The presence of useless mitochondria in dopaminergic neurons has been connected to the development of PD. The way that various qualities related with familial PD debilitate mitochondrial homeostasis firmly recommends that mitochondria assume a critical part over PD as shown in Figure 2.1 (Borsche et al., 2021).

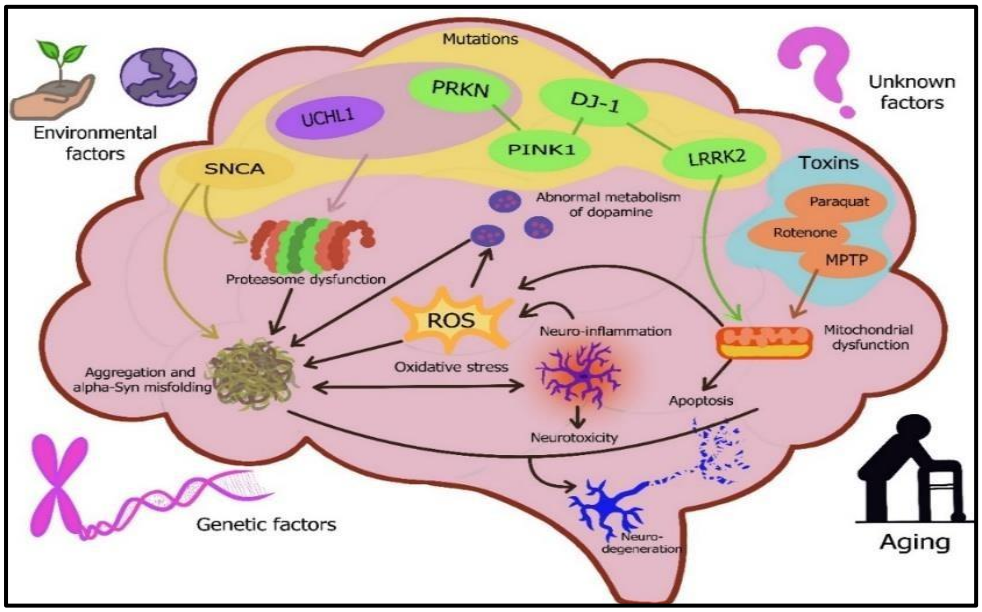


Figure 2.1: Pathogenesis of PD. Depicting various cellular and molecular mechanisms such as mitochondrial dysfunction, neuroinflammation, and α-synuclein aggregation.

2.5 Etiology

PD emerges from hereditary, natural, and organic factors. While the exact reason for PD is at this point unclear, broad review has yielded significant information about the few components that assume a part in the turn of events and headway of the disease. The primary variables adding to the development of PD are presented in Figure 2.2

1. Genetic factors: PD can be passed down via families or develop spontaneously, and both kinds are influenced by genetic predisposition. It is assessed that around 5 to 10% of PD cases have an inherited part. A few genes, including SNCA, LRRK2, PARKIN, PINK1, and DJ-1, have been connected to familial PD, alongside hereditary variations in these qualities (Poewe et al., 2017).

2. Environmental factors: The probability of affecting with PD is increased when persons are exposed to certain environmental circumstances. Both Paraquat and rotenone, which are delegated the two pesticides and herbicides, have been connected to an expanded weakness to develop PD. Other potential environmental risk factors encompass heavy metals, industrial pollutants, and contamination in well water (Blesa & Przedborski, 2014).

3. Age: Age-related variables are a significant contributing factor in developing PD. The decrease in cellular repair processes linked to ageing may contribute to the buildup of cellular damage.

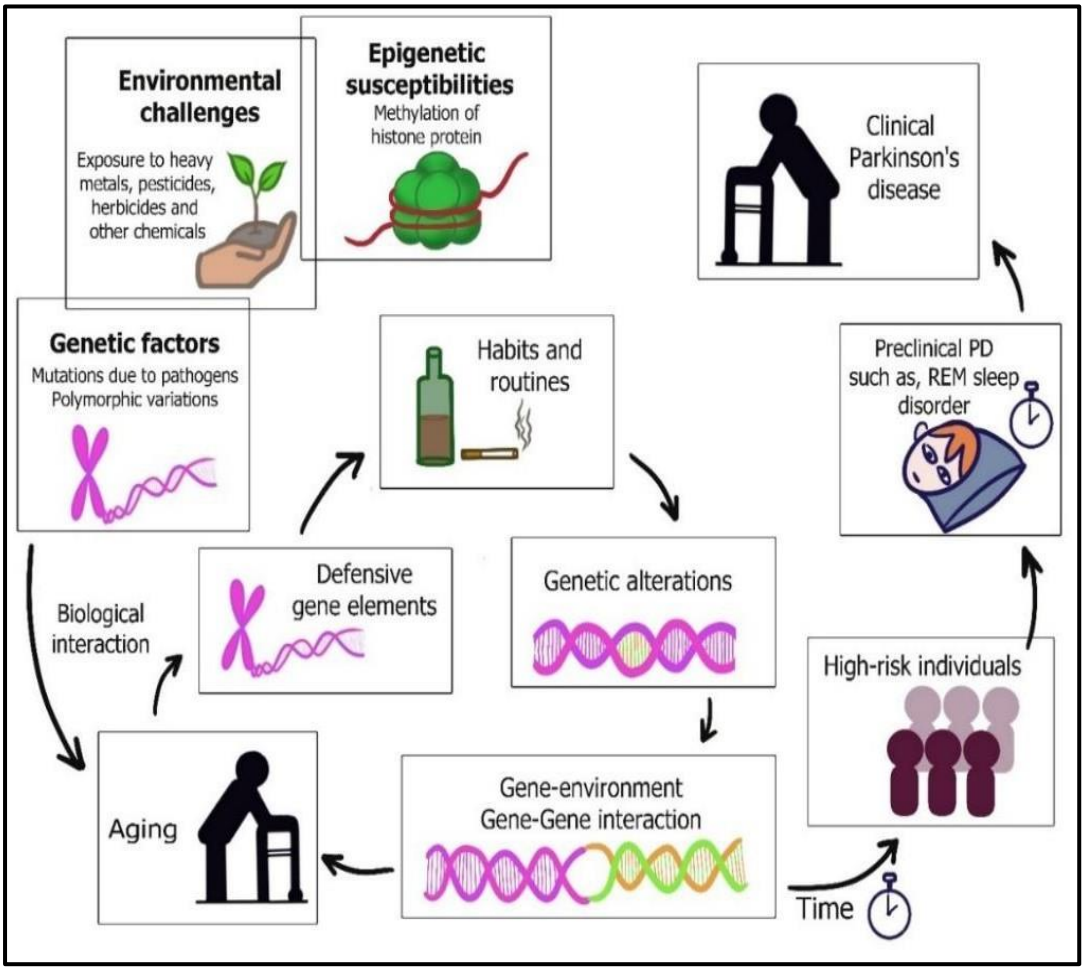


Figure 2.2: Etiologies of PD. Shows interaction between genetic, epigenetic, and environmental factors influencing PD.

2.6 Management and treatment

2.6.1 Pharmacological interventions

At now, there are no medications that can alter the course of PD. Nevertheless, the current medications can successfully reduce the motor symptoms and offer significant relief.

2.6.1.1 Levodopa

Levodopa, alluded to as L-dopa, is the best medication for PD. A compound can be switched into dopamine and has the limit to cross the defensive boundary between the blood and the brain. When it enters the mind, it is changed into dopamine by the

enzymatic activity of aromatic L-amino acid decarboxylase (AADC). Levodopa is every now and again utilized as the essential treatment for PD and has demonstrated to be especially viable in diminishing motor-symptoms, for example, bradykinesia, stiffness, and tremor (Zahoor et al., 2018).

Levodopa is commonly combined with carbidopa or benserazide to inhibit its peripheral metabolism by AADC, so increasing the amount of levodopa that reaches the brain and reducing unwanted side effects including nausea and vomiting (Weintraub et al., 2008).

2.6.1.2 Dopamine agonists

Dopamine agonists are as follows,

- Pramipexole is a selective dopamine agonist that binds only to D3 receptors and does not fall into the category of ergot medicines (Poewe et al., 2017).
- Ropinirole is a dopamine agonist that targets D2 and D3 receptors, and it does not belong to the ergot class of drugs (Calabresi et al., 2006).
- Rotigotine is a non-ergot dopamine agonist that is conveyed by a transdermal fix, empowering the steady conveyance of drug
- Apomorphine is a potent and rapid-acting medication that activates dopamine receptors. It is frequently employed to promptly relieve off episodes (Furth et al., 2013).

Adjunct therapy involves the use of additional medications with levodopa to manage motor fluctuations and reduce Dyskinesias in persons with severe PD. Dopamine agonists can eliminate antagonistic impacts like sickness, orthostatic hypotension, visualizations, and drive control issues including obsessive betting and impulsive purchasing (Chakravarty et al., 2022).

2.6.1.3 MAO-B inhibitors

MAO-B inhibitors hinder the movement of the protein MAO-B, liable for the debasement of dopamine in the mind, thus expanding the span of dopamine's belongings (Calabresi et al., 2006).

- Selegiline functions as an MAO-B inhibitor and has a minor symptomatic impact.The substance undergoes metabolism to form amphetamine derivatives, which might potentially contribute to its effects.
- Rasagiline is an exceptionally powerful and explicit inhibitor of the protein monoamine oxidase-B (MAO-B), and it doesn't create metabolites that are like amphetamines.

Monotherapy is employed during the initial phases of PD to offer moderate alleviation from symptoms. Adjunct therapy refers to the use of additional treatment alongside levodopa to extend its therapeutic benefits, hence assisting in the management of motor fluctuations (Chaudhuri & Schapira, 2009).

2.6.2 Deep brain stimulation

Deep Brain Stimulation (DBS) has arisen as a significant progression in the domain of restorative neurosciences in the beyond twenty years. The DBS, filling in as a careful surgical device, may assess abnormal mind capability and convey customized feeling to create restorative impacts in neurological and mental issues connected to useless brain processes. DBS is a careful tool that involves the placement of sensors in unambiguous locales of the mind, for example, the globus pallidus internus (GPi) or subthalamic core. These gadgets change unusual brain action and mitigate motor debilitations by conveying electrical driving forces. DBS is oftentimes proposed as a

treatment choice for people who experience motor dysfunctions and display a great reaction to levodopa prescription (Lozano et al., 2019).

2.6.3 Non-pharmacological therapies

Non-pharmacologic interventions can contribute to the overall well-being of individuals with PD, but they may not directly affect the major symptoms of the condition. Counseling and cognitive-behavioral therapy (CBT) are frequently used to treat non-motor symptoms including anxiety and depression. Participating in exercises specifically focus on enhancing flexibility, strength, and balance and sustain equilibrium, and actively engage in routine tasks. Vocal training is a focused method used to specifically tackle issues related to voice and speech (Schapira et al., 2014).

2.7 Gut brain axis

The Gut-Brain Axis (GBA) is an arrangement of two-way correspondence between the central nervous system and the peripheral nervous system (Fitzgerald et al., 2019). Increasing evidence suggests that the microscopic organisms in the gut might communicate with parts of the human neuroendocrine framework. Also, the gut microbiota produces numerous chemical like mixtures that arrive at the circulation system and apply their impacts on far off areas and organs, including the brain. The neurological and endocrine frameworks are firmly interconnected and mutually direct a huge number of physiological capabilities in the human body (Cussotto et al., 2018). Also, they have been related with different mental and neurological circumstances, for example, PD, multiple sclerosis, and ongoing agony (Fitzgerald et al., 2019).

Healthy gastrointestinal microbiota plays a part in keeping up with the blood brain barrier (BBB) by managing the tight intersection proteins, for example, occludin and

claudin-5, through the activity of short chain unsaturated fats (SCFAs). Short-chain unsaturated fats (SCFAs) have a pivotal capability in saving the uprightness of the digestive boundary by restraining the development of microorganisms over it. This cycle is connected to the event of irritation in the digestive organs, as well as aggravation all through the body and in the sensory system. α-syn is plentifully present in the mind and exists in the enteric nervous system (ENS), where it is synthesized by intestinal neurons to work with the delivery and assimilation of synapses. Pathological α-syn aggregates have been discovered in gastrointestinal (GI) tissue biopsies of persons with PD (Cussotto et al., 2018). Significantly, α-synuclein has also been detected in the salivary glands, esophagus, and stomach (Fayyad et al., 2017), which may be associated with typical non-motor symptoms such excessive salivation, difficulty swallowing, delayed stomach emptying, and gastroparesis (Klann et al., 2022).

2.7.1 Hypothalamic-Pituitary-Adrenal (HPA) axis

The hypothalamic-pituitary-adrenal (HPA) axis is a noticeable neuroendocrine framework inside the human body. At the point when there is an outside excitement, neuroendocrine neurons in the periventricular nucleus (PVN) of the hypothalamus produce and deliver vasopressin and corticotrophin-delivering factor (CRF). These two peptides upgrade the arrival of adrenocorticotropic chemical (ACTH) from the corticotrophic cells of the pituitary organ (Fayyad et al., 2017). The arrival of ACTH prompts the creation of glucocorticoid chemicals by the adrenal cortex. In humans, this hormone is cortisol, whereas in rodents it is corticosterone. These two hormones thereafter have a negative feedback effect on the brain and pituitary gland. The HPA pivot fills in as the essential controller of the body's pressure reaction. Furthermore, it

plays a part in directing a few physical processes including absorption, the safe framework, mind-set, feelings, sexuality, and energy use. At the point when a living being sees a danger, the HPA pivot starts a guarded response to reestablish homeostatic equilibrium. The feeling of the HPA pivot at last prompts the arrival of conduct adjusting glucocorticoids, mineralocorticoids, and catecholamine. The capability of the HPA hub is constrained by numerous tangible thoughtful, parasympathetic, and limbic pathways (like the amygdala, hippocampus, and prefrontal cortex) that give immediate contribution to the PVN (Sheng et al., 2021).

2.8 Rosmarinus officinalis

Rosmarinus Officinalis, often known as rosemary, is a perennial plant with a woody stem. It is frequently employed as a seasoning and has a broad spectrum of therapeutic uses. It is also recognised for its ability to stimulate, provide modest pain relief, stimulate bile production, have anticancer effects, and preserve the liver (Andrade et al., 2018). These chemicals have properties that reduce inflammation, counteract oxidative stress, and inhibit the development of cancer. This medicinal plant is renowned for its therapeutic capabilities, which encompass its capacity to address ailments such as inflammation, cancer, diabetes, blood coagulation, oxidative stress, liver protection, neurological protection, and relief from depression. *R. officinalis* has attracted significant attention in the field of herbs and spices due to its abundant presence of phytochemicals such as CA, RA, UA (Shao et al., 2018).

2.8.1. The bioactive components of *R. officinalis* and their therapeutic potential

Rosmarinus officinalis, often known as rosemary, has many bioactive compounds that may offer therapeutic benefits for PD (Figure 2.3). CA and ursolic acid (UA) are well-known components that possess strong antioxidant and anti-inflammatory properties. CA further improves the Nrf2 pathway, expanding the combination of cancer prevention agent compounds that safeguard neurons from oxidative harm. Studies indicate that CA and carnosol has the capacity to protect dopaminergic neurons (de Oliveira et al., 2019). RA, an essential component, possesses strong antioxidant activity, effectively neutralizing free radicals and protecting against oxidative damage. Furthermore, it impedes the production of molecules that stimulate inflammation and regulates the activity of chemical messengers in the brain, thus restoring the balance that is disrupted in PD. Studies have shown that RA protects neurons from various forms of neurotoxicity, improves cognitive function, and reduces symptoms associated with neuroinflammation. UA, present in rosemary, exhibits significant antioxidant effects via decreasing lipid peroxidation and oxidative stress. Furthermore, it assists in safeguarding the neurological system by inhibiting the expression of genes that stimulate inflammation. The bioactive constituents of rosemary have huge commitment as neuroprotective specialists. They can possibly alleviate oxidative pressure and neuroinflammation, both predominant in PD. *R. officinalis* can possibly be utilized as a restorative treatment to control and maybe decelerate the progression of the infection successfully (Andrade et al., 2018).

Figure 2.3 Dried leaves of *R. officinalis*

2.9 Bacillus Clausii

Bacillus clausii is a probiotic bacterium. It is widely recognised for its capacity to regulate the immune system and decrease inflammation. These characteristics have gain interest in the evaluation of neurodegenerative issues including PD (Acosta-Rodríguez-Bueno et al., 2022). *B.clausii* possesses the capacity to rectify the equilibrium of bacteria in the gastrointestinal tract, diminish inflammation in the intestines, and enhance the functionality of the gut barrier. This can aid in the reduction of inflammation in both the body and the brain system, which is linked to PD. Furthermore, *B.clausii* produces short-chain fatty acids (SCFAs) that can effectively control immunological reactions and reduce oxidative stress, leading to neuroprotective advantages. Although there is limited direct evidence linking *B. clausii* to the treatment of PD, the probiotic's beneficial effects on gut health and immune regulation present a compelling case for its potential in alleviating PD symptoms and potentially slowing

down disease progression. Further clinical trials are necessary to authenticate these benefits and elucidate the underlying mechanisms (Paparo et al., 2020).

2.10 MPTP- induced Model of Neurotoxicity

MPTP is the best model for examining the systems behind the degeneration of dopamine neurons in PD. MPTP has shown toxicity across a wide scope of animal types. Notwithstanding primates, mice are usually utilized because of rodents' resistance to this toxin (Hirsch & Hunot, 2009). Over the years, several methods of intoxication have been used in both mice and primates. The two species experience significant mischief to the nigrostriatal dopamine pathway because of MPTP, bringing about a huge decrease in dopamine levels in the striatum and substantia nigra pars compacta (SNpc). The principal benefit of this worldview is its steady and exact ability to initiate a neurotoxic effect on the nigrostriatal pathway. Neuropathological data shows that injecting MPTP causes damage to the nigrostriatal dopamin pathway, which is identical to the damage seen in PD. Very much like in PD, MPTP causes a more noteworthy decrease in dopamine neurons in the SNpc contrasted with the ventral tegmental region (VTA) or the retrorubral field (Blesa & Przedborski, 2014).

MPTP is a favorable to poison fit for crossing the BBB. Subsequent to entering the mind, it goes through a change into its dynamic state known as MPP+, or 1-methyl-4-phenylpyridinium, by the activity of the catalyst monoamine oxidase B (MAO-B) tracked down in astrocytes. MPP+ is then taken up by dopaminergic neurons through the dopamine transporter (DAT) and disturbs the activity of mitochondrial complex I, prompting diminished mitochondrial capability, raised oxidative pressure, and resulting neuronal passing as shown in Figure 2.4 This approach copies the exact weakening of dopaminergic neurons in the SNpc, which is an unmistakable quality of PD. Thus, there

are huge decreases in dopamine levels in the striatum, prompting motor symptoms, for example, bradykinesia, stiffness, and postural unsteadiness. Mice exposed to MPTP treatment display motor hindrances, which are surveyed utilizing social assessments including the open field test, cylinder test, and post test (Presti-Silva et al., 2023). Moreover, the MPTP model shows expanded degrees of oxidative stress markers and neuroinflammatory responses (Jackson-Lewis & Przedborski, 2007).

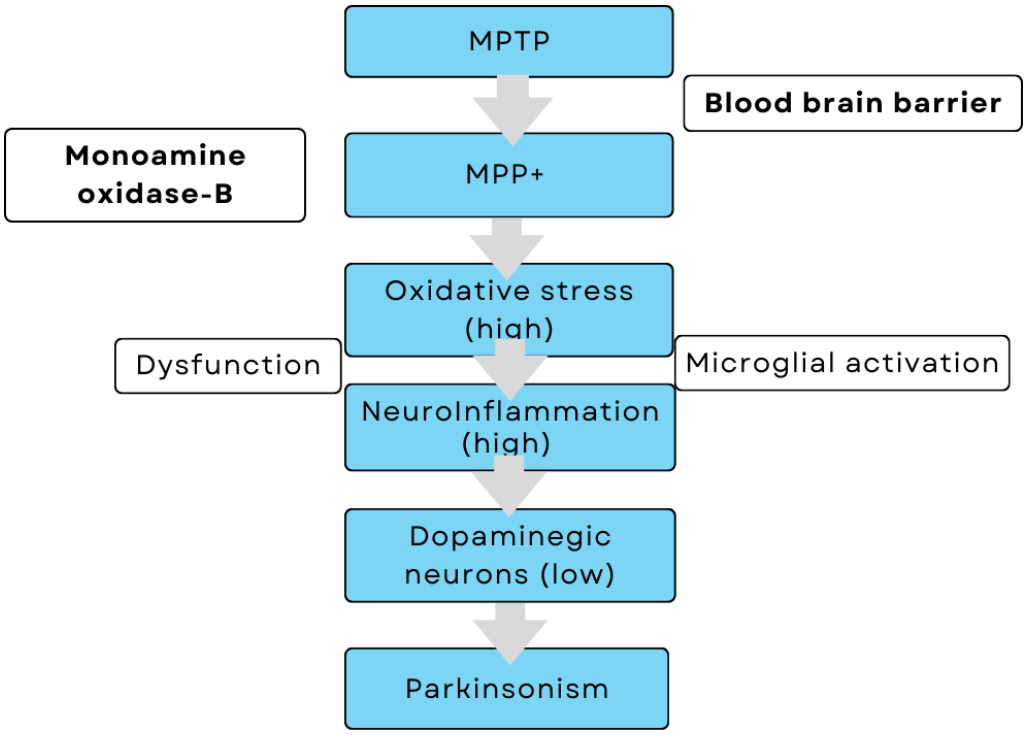


Figure 2.4 Mechanism by which MPTP and MPP+ contributes to the advancement of PD.

The outline portrays the quick development of the toxic metabolite of MPP+ by the activity of monoamine oxidase-B on the lipophilic particle MPTP and it can cross BBB. MPP+ specifically frustrates the role complex I in mitochondrial electron transport chain by solely entering dopaminergic neurons by means of dopamine carriers. This results in the manifestation of PD.

2.11 Computational methods for drug development

The insilico approach is regularly utilized in the main phases of prescription advancement for neurodegenerative disorders (NDDs). The Insilco approach is very efficient and cost-effective for forecasting the most promising therapy choices for various NDDs. Additionally, it significantly reduces errors during the final phases (Makhouri & Ghasemi, 2018). Molecular docking simulations are widely employed in computer-aided drug design due to their capacity to predict molecular-level interactions between receptors and ligands (Liu et al., 2022). As a result, they make it easier to find new potential treatments in a short amount of time. In silico approaches facilitate wet lab research by generating a roster of probable candidates, hence diminishing the expenses associated with medication development.

Moreover, a reliable assessment of docking is associated with a more accurate functioning of the employed scoring system in determining the mode and position of ligand binding, predicting the binding affinity, and identifying potential top candidates for a specific target protein. Over the past several years, numerous software tools have been developed specifically for molecular docking. These technologies utilize a range of algorithms and physicochemical approximations. Auto Dock Vina is an often cited and open-source application that has demonstrated excellent docking skills. In addition, it showed a higher capacity to accurately assess the strength of binding compared to 10 regularly utilized docking programs (Wang et al., 2016).

However, it has significant limitations and has been seen to have challenges in reliably detecting and analyzing the crystal structures of ligands in benchmark research. Moreover, it does not have the capacity to replicate certain characteristics such as macro cycles or explicit water molecules. The latest revisions of Auto Dock Vina 1.2.0 have resolved a functional constraint by including the scoring mechanism from Auto Dock

4.2 (Eberhardt et al., 2021). Adding a rectification term to the scoring capability works on the accuracy of protein-ligand docking and screening. The upgrade altogether further develops the forecast abilities of Auto Dock Vina for docking and screening position, as confirmed by its presentation on CASF-2016, DUD-AD, and DUD-E (Holcomb et al., 2023).

Materials and Methods

3.1 Ethics statement

The experiments were carried out in accordance with the NIH Guide for Care and Use of Laboratory Animals: 8th Edition, 2011 and were granted approval by the Internal Review Board of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan (2024-IRB-A-10/10). All the animals were bred and kept in a constant environment in the animal house of ASAB, NUST.

3.2 Animals

The study used 64 male BALB/c mice aged 6-7 weeks with an average weight of 25-30 g. The animals were randomly divided into eight groups, each consisting of eight mice. Mice were kept in cages of 40 cm x 25 cm x 15 cm in a temperature-controlled setting (22 ± 2 ℃), with a regular light-dark cycle. They had full access to water and food.

3.3 Drugs and Chemicals

MPTP was purchased from AK Chemicals, China (CAS no: 23007-85-4) and stored at 2-8°C. Lyophilized pre-packaged probiotic formulation of *Bacillus clausii,* which is sold under the brand name Ospor and is manufactured by Matrix Pakistan (Pvt) Ltd, was acquired from drugstore in Islamabad. *R. officinalis* leaves were acquired from local market in Rawalpindi. Necessary reagents for gene expression analysis were obtained from Sigma Aldrich.

3.4 Extraction protocol of *Rosmarinus officinalis*

The *R. officinalis* extract was prepared from dried leaves using the methodology as described previously (Mirza et al., 2021).

Essentially, a 1:10 proportion of solute to solvent was used for the extraction process. The desiccated leaves were initially pulverized into a fine powder. To ensure consistent particle size following the grinding process, the powder was filtered using 80 mesh sieves. Afterwards, a quantity of 10 gm of the powdered substance was put inside a thimble. The thimble was then inserted into a Soxhlet extractor device along with a round-bottom flask that contained 100 ml of 100% ethanol. The extraction technique went on for 24 hours. The concentrate got dried out utilizing a revolving evaporator at a strain of 68°C, and afterward put away at 4 °C.

3.5 Study design

An experimental method lasting 28 days was made, including 64 mice that were separated into 8 groups, with 8 mice in each groups (n=8 each). The mice were first acclimatize for 5 days to get possessed to the climate and feed.To cause neurotoxicity, four out of the eight groups were subjected to MPTP intraperitoneally consecutive for 05 days. After a 5-day period of exposure, the mice were treated on the 16th day (Post 5 days of MPTP administration) by administering Probiotic orally and RE intraperitoneally (i.p) for 7 days. In this way, behavior test battery were directed to assess the effect of treatment on locomotor movement, bradykinesia and motor impairments. On the 28th day, mice were beheaded, and brain samples were gathered for histopathological evaluation (Figure 3.1).

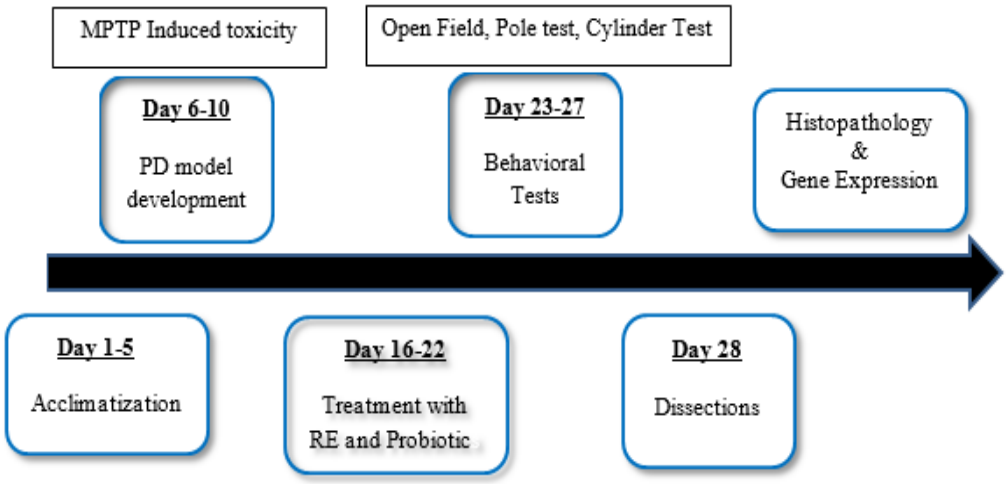


Figure 3.1 Study Design

3.6 Animal Treatment

There were a total of 64 healthy mice, all of which were 6-7 weeks old, divided into 8 groups, with each groups comprising of 8 mice. The groupings are described in Table 3.1.

No.	Groups	Sub-groups (n=8)	Treatment	
			5 days	7 days
1	Controls	Control	Distilled H_2O	Normal saline
		R. officinalis	Distilled H_2O	*R.officinalis* extract (RE)
		B.clausii	Distilled H_2O	*B.clausii*
		R. officinalis + *B.clausii*	Distilled H_2O	*R. officinalis* extract (RE) + *B.clausii*
2	Diseased	MPTP	30 mg/kg MPTP (IP injection)	Normal saline
		MPTP+ *R. officinalis*	30 mg/kg MPTP (IP injection)	*R. officinalis* extract l20 mg/kg (IP injection)
		MPTP + *B.clausii*	30 mg/kg MPTP (IP injection)	*B.clausii* 2*10^4 CFU (Oral)
		MPTP+ *R. officinalis* + *B.clausii*	30 mg/kg MPTP (IP injection)	*R. officinalis* extract 120 mg/kg+ *B.clausii* 2*10^4 CFU

Table 3.1: Grouping of animals for inducing PD following drug treatments

3.7 Behavioral studies

After developing the PD model, behavioral tests were carried out to determine any motor function impairments. Three tests were conducted i.e. the Open Field test, Pole test, and Cylinder test.

3.7.1 Open field test

The Open Field Test is a commonly employed technique for evaluating an animal's capacity to explore the field and its locomotor activity (Horka et al., 2024). The Open Field Test serves as a direct sensorimotor assessment designed to measure overall activity levels, large-scale movement, and exploratory tendencies. The assessment employs a square layout for testing. The test participants, specifically mice in this instance, are positioned in one of the corners of the square, and their actions are monitored for 10 minutes (Kraeuter et al., 2019). The count of filled squares, as well as the animal's investigation of the outer squares adjacent to the wall and the inside squares, are tallied individually.

3.7.2 Pole Test

The pole test assesses the slowness of movements, and prolonged latency to descend is indicative of motor impairment. During the Post Test, a mouse is situated on the highest point of a pole with its head confronting upwards and is told to drop to the base ceaselessly. The animal's capacity to drop to the floor by rotating its body without losing balance is employed to evaluate its locomotor function or dysfunction. The Pole Test needs just basic equipment and yields immediate outcomes. Additionally, the test might be performed within the animal's home enclosure as it may have a preference for descending to a familiar area (Matsuura et al., 1997).

3.7.3 Cylinder Test

The cylinder test surveys the mice's deliberate utilization of its front appendages, which might be used to assess the forelimb asymmetry and rearing events of mice . During this trial, the mouse is put inside a straightforward cylinder and the recurrence of its upward developments, when it remains on its rear legs and contacts the cylinder wall, is recorded. An observer in slow movement records the wall contacts for the left, right, or the two paws. Notice the mice for a span of 10 minutes. After the test, quickly return the mouse to its home enclosure. The findings are shown as the percentage of paw usage for each paw, compared to the total number of touches (Magno et al., 2019). The cylinder test was calculated by the formula:

[(contralateral touches)/(ipsilateral touches + contralateral touches) x 100]

A ratio of 0.5 indicates equal usage frequency of both forelimbs, whereas a score below 0.5 suggests motor impairment of the contralateral forelimb.

3.8 Statistical analysis

The data was statistically assessed through GraphPad Prism 10.0.3. One-way anova (ANOVA) was utilized to decide measurable significance inside the dataset. Bonferroni's Multiple comparison test was then used to recognize explicit contrasts between groups. Error bars were addressed as Mean ± SEM. A *P* value <0.05 was viewed as significant.

3.9 Brain Dissection

The mice were euthanized using the cervical dislocation procedure. Initially, the mice were subjected to anesthesia using chloroform. The head was delicately moved forward, and an incision was created behind the ears using surgical scissors. A little surgical cut was performed starting at the bottom of the head at the parietal bone. Two more incisions were made on the occipital bone. Subsequently, the parietal bone on both sides was inclined and fractured to uncover the brain, and the frontal bone was also incised to delicately extract the brain.

Subsequently, the brain was positioned on a cooled metal tray, with the ventral side of the brain facing downwards, and was meticulously cleansed using pre-chilled Phosphate Buffer Saline (PBS). After completing the cleaning process, the olfactory bulb and cerebellum were surgically removed using a knife. Subsequently, a pair of small-curved forceps was positioned in a closed state between the cerebral halves, and then they were carefully opened to expose the cortical halves. After achieving an adequate gap in the centre line, the forceps were rotated both anticlockwise and clockwise at 30-40° to detach the left and right hippocampus from the cortex, respectively. The brain and hippocampus were thereafter placed into a pre-cooled Eppendorf tubes and kept at -80 °C.

3.10 Histological examination

3.10.1 Hematoxylin and eosin staining

The mouse was anaesthetized using chloroform, and then the entire brain was removed. The brain was rinsed with PBS and promptly immersed in a 4% Paraformaldehyde (PFA) solution 24 hours prior to subsequent procedures. Following a 24-hour incubation in a 4 % PFA solution, the brain tissue was dehydrated using 70%, 95%, and

100% isopropanol for one hour each before paraffin infusion. The tissue was then submerged in xylene for 4 hours. Afterwards, the tissue was placed in liquid paraffin and left to be imbedded for 4 hours at 60 °C. Afterwards, the sample was let to solidify inside the mould at 4 °C, prior to being cut into slices.

The tissue samples underwent Mayer's hematoxylin staining for 8 minutes, followed by a 10-minute washing with warm water. Subsequently, a counterstaining operation was performed by administering eosin for 30 seconds. After a final 10-minute rinse with warm water, the slides were left to air dry and then secured with a cover slip for examination under a microscope (Optika, Italy) by using software Optika lite 9.0.

3.11 Gene expression analysis

The Trizol technique was utilized to extract RNA, as indicated by producer's guidelines. The materials were pulverized and homogenized utilizing 1ml of Trizol, and afterward kept on ice for 10 minutes. Then, 200 µl of chloroform for each 1 ml of Tri-reagent were added, and the samples were mixed for 15 seconds, trailed by a 10-minute resting stretch. The samples were exposed to centrifugation for 15 minutes at 4°C at 12,000 rpm. Following centrifugation, the top aqueous phase, which contained the RNA, was transferred to a separate clear Eppendorf tube. For making the RNA separate out of solution, 500 µl of isopropanol was added to each sample and left to go through incubation for 10 minutes. A short time later, the samples went through centrifugation for 15 minutes at 12,000 rpm at 4 °C. The liquid portion was extracted cautiously, and the RNA solid residues were rinsed with 1 ml of pure ethanol. To wipe out any leftover ethanol, the examples were exposed to one more round of centrifugation at a speed of 7,500x for 5 minutes at 4 °C. The pellet was dried and afterward re-suspended in 30 µl of nuclease free water. It was then kept at a - 80 °C.

3.11.1 Quality of RNA

The RNA conc. in each example was rmeasured utilizing the NanoDrop procedure, and the nature of the extracted RNA was evaluated in light of the A 260/280 proportion. The A 260/280 ratio was within the range of 1.8 to 2.0, suggesting high RNA quality.

3.11.2 cDNA synthesis

The quantification of RNA was conducted using the Bio photometer Plus (Eppendorf, Germany). Each reaction consisted of 4 µg of RNA. While the reaction mixture consisted of 2 µl of 10 mM dNTPs, 4 µl of 5X RT buffer, 1 µl of 10 mM oligo-dT, and 1µl of Revert AID enzyme. Subsequently, PCR water was added to attain a final reaction volume of 25 µl. The precise thermocycling parameters for the cDNA synthesis is provided in Figure 3.2.

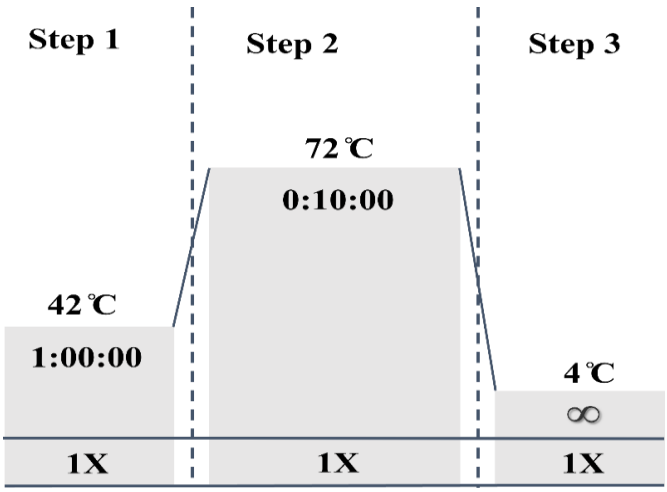


Figure 3.2 Thermocycling conditions for cDNA synthesis

3.11.3 Quantitative-real time PCR for Gene expression analysis

Gene expression study was directed utilizing (quantitative Polymerase Chain Response) qPCR with a SYBR Green fluorescent dye eaction mixture . The reaction mixture involved 1 µl of both the forward and reverse primers, 4 µl of SYBR Green color, and 1 µl of the cDNA. Nuclease free water was added to get a last volume of 20 µl. The samples were analyzed in duplicate and afterward standardized utilizing the β-actin reference gene. The thermocycling settings used for gene expression analysis were as per the following: The cycle includes an underlying pre-incubation at 95 ℃ for 5 minutes, trailed by a denaturation time of 30 seconds.The extension stage comprised of 35 cycles, each cycle comprising of 30 seconds of denaturation at 95 °C, 1 minute of annealing tempering at 60 °C, and 30 seconds of extension at 72 ℃ as displayed in Figure 3.3. The stage finished up with a last denaturation step. The exact successions of the RT-PCR introductions for the qualities are referenced in Table 3.2.

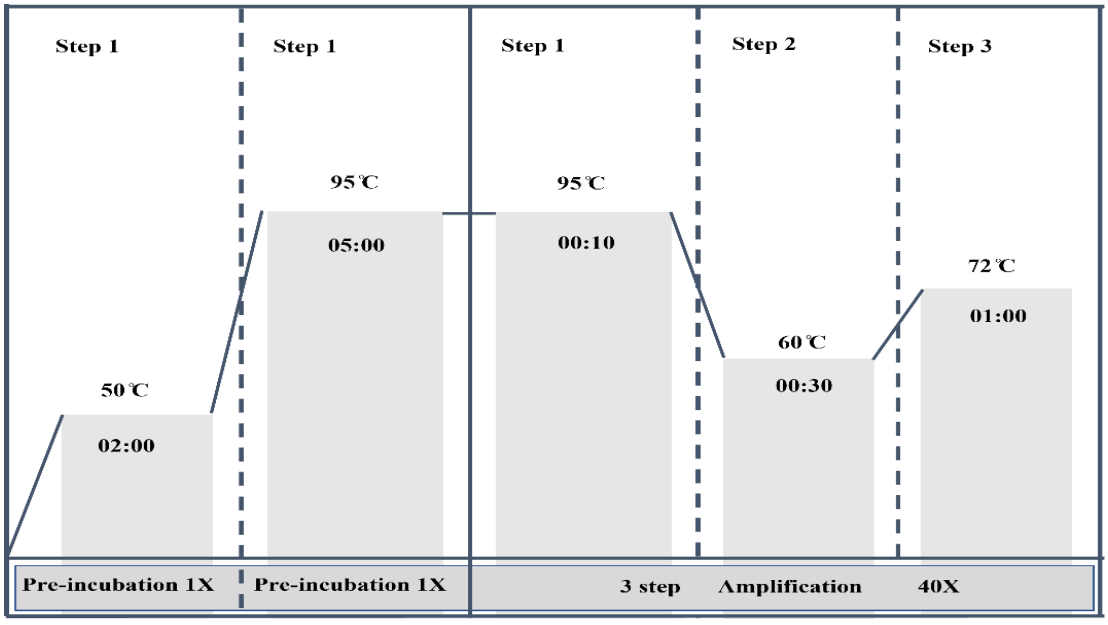


Figure 3.3: Real Time qPCR profile for gene expression quantification of SOD1 and SOD2, IL-6, TNF-α.

Primer		Sequence
β-Actin	Forward	5′-GCCTTCCTTCTTGGGTATGG-3′
	Reverse	5′-CAGCTCAGTAACAGTCCGC-3′
IL-6	Forward	5′-AGACAGCCACTCACCTCTTCAG -3′
	Reverse	5′-TTCTGCCAGTGCCTCTTTGCTG-3′
TNF-α	Forward	5′- ATGAGCACAGAAAGCATGA -3′
	Reverse	5′- ACCACGCTCTTCTGTCTACT -3′
SOD1	Forward	5′- GACAAACCTGAGCCCTAAG-3′
	Reverse	5′- CGACCTTGCTCCTTATTG-3′
SOD2	Forward	5′-ATGTCTGTGGGAGTCCAA-3′
	Reverse	5′-TGAAGGTAGTAAGCGTGCTC-3′

Table 3.2 Primer sequences

3.12 In silico analysis

3.12.1 Structural analysis of SNCA, PRKN, APOE ,IL-6, TNF-α, SOD-1 and SOD-2

Molecular interactions of RA, CA, UA, Carnosol, Genkwanin, Camphene, limonene and Levodopa with SNCA, PRKN, APOE, IL-6, TNF-α, SOD-1 and SOD-2 were tested. The 3D structures of the SNCA (PDB ID: 1XQ8), PRKN (PDB ID: 5TR5), APOE (PDB ID: 6V7M), IL-6 (PDB ID: 1IL6), TNF-α (PDB ID: 1TNF), SOD-1(PDB ID:2RSQ) and SOD-2(PDB ID: 1EN5) were acquired and downloaded from RCSB Protein Data Bank (PDB) (http://pdb101.rcsb.org/motm) (accessed on Jan 20,2024) by

applying refinements of *Homo Sapiens* and proteins on source organism and polymer entity type respectively. For the removal of bound ligands and all non-standards with the target proteins, UCSF Chimera 1.14 was used (Pettersen et al., 2004) (https://www.cgl.ucsf.edu/home/tef/pubs/chimera.pdf.) The isolated structures of SNCA , PRKN, APOE , IL-6, TNF-α, SOD-1 and SOD-2 were obtained and then saved as a PDB file.

3.12.2 Ligand retrieval

The 3-D conformer structures of the ligands selected from extensive literature review for the study; Levodopa (CID: 6047), CA (CID: 65126), RA (CID: 5281792), Carnosol (CID: 442009), Genkwanin (CID: 5281617) Limonene (CID: 22311), Camphene (CID: 6616), UA (CID: 64945); were retrieved from chemical database PubChem (https://pubchem.ncbi.nlm.nih.gov/) (accessed on 22 Jan, 2024) and their structures were downloaded in SDF file format.

3.12.3 Binding site detection for defining binding pocket

The PDB IDs for SNCA (1XQ8), PRKN (5TR5), APOE (6V7M), IL-6 (1IL6), TNF-α (1TNF), SOD-1 (2RSQ), SOD-291-ENF) were searched on the ProteinsPlus website (https://proteins.plus/) (Schöning-Stierand et al., 2020). The DoGSiteScorer (https://bio.tools/dogsitescorer) (accessed on Jan 21, 2024) (Volkamer et al., 2012) function was employed to find binding sites, using a grid-based approach that identifies probable binding pockets based on the 3D structure of the protein. The best binding pockets, which were more druggable, were selected based on the greatest drug score value (close to one). The amino-acid residues were then recorded for the purpose of conducting molecular docking.

3.12.6 Evaluation of drug likeness and pharmacokinetic parameters

The canonical smiles of RA, CA, and UA, together with their corresponding PubChem Compound Identifiers (CIDs), were obtained from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) (accessed on Feb 12, 2024). These canonical grins are then used to establish the therapeutic compatibility and pharmaceutical (Absorption, Distribution, Metabolism, and Excretion) ADME qualities. The Lipinski rule of five is employed to assess the drug-like characteristics. The Lipinski filter employs a set of five rules to forecast the drug's compliance. Orally active medication must adhere to four out of five requirements. The criteria consist of hydrogen bond acceptor, hydrogen bond donor, molecular mass, logP, and molar refractive index.

SwissADME and admetSAR1 are utilized to assess the characteristics. SwissADME can be accessed at (http://www.swissadme.ch/) (accessed on Feb 12, 2024) and admetSAR1 can be accessed at (http://lmmd.ecust.edu.cn/admetsar1/predict/?) (accessesd on Feb 13, 2024) Smiles=&actio. Canonical smiles are utilized to get the necessary information from these tools.

Results

4.1 Assessment of locomotor activity

The open field test was performed to evaluate the impact of *R. officinalis* and *B. clausii* on locomotor activity in MPTP-induced PD mice model. The number of boxes crossed per 10 minutes in both the central and peripheral regions of the field was recorded. MPTP-treated mice demonstrated significant reduction in locomotor activity compared to the control group.

In the central region, MPTP treatment significantly reduced the number of boxes crossed compared to the control group ($p < 0.0001$; 7.167±0.8692). However, treatment with *R. officinalis* ($p < 0.0001$; 9.333±0.8692) and *B. clausii* ($p < 0.0001$; 7.833±0.8692) significantly improved locomotor activity, when compared to the MPTP-treated groups. The combination of *R. officinalis* and *B. clausii* exhibited the greatest recovery ($p < 0.0001$; 10.33±0.8692).

In the peripheral region, MPTP-treated mice displayed significantly reduced locomotor activity compared to the control group ($p < 0.0001$; 34.33±1.196). Treatment with *B. clausii* alone led to a significant improvement ($p < 0.0001$; 28.50±1.196), while treatment with *R. officinalis* demonstrated a substantial recovery in motor function ($p < 0.0001$; 25.50±1.196). The combined treatment of *R. officinalis* and *B. clausii* also showed significant effectiveness ($p < 0.0001$; 24.33±1.196) (Figure 4.1).

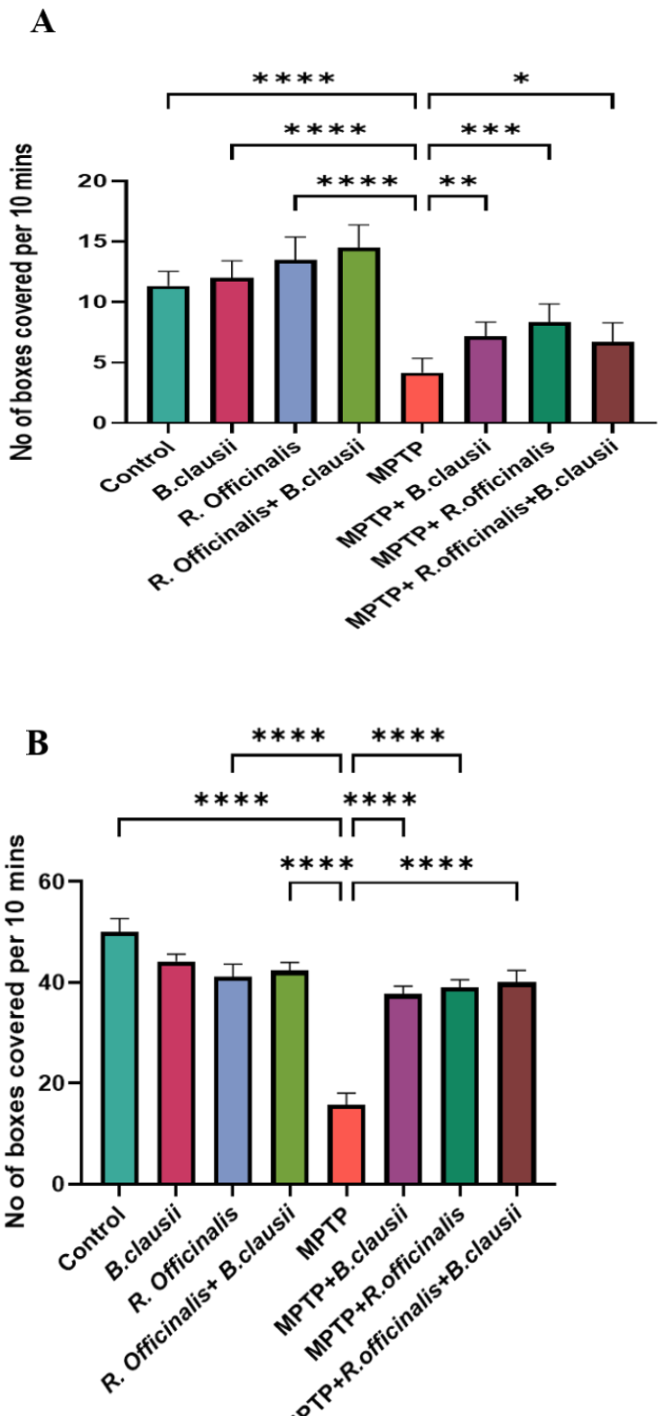


Figure 4.1 Locomotor activity was evaluated using the Open Field Test, (A) Central and (B) peripheral regions. Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni *post hoc* test to compare mean values with the standard error of the mean (Mean ± SEM). The sample size was n=6, and significance levels were indicated as ****p<0.0001,***p<0.001,and**p<0.01.

4.2 Effect of *R. officinalis* and *B. clausii* on Bradykinesia

Bradykinesia, a characteristic symptom of PD, was evaluated using the pole test in MPTP-induced PD mice model. The pole test assesses the slowness of movements, and prolonged latency to descend is indicative of motor impairment. Treatment with *R. officinalis* and *B. clausii* was investigated for its potential to improve bradykinesia in MPTP induced PD mice.

MPTP exposure significantly increased the latency to descend the pole, indicating motor deficits characteristic of PD (MPTP -treated groups: 16.67 ± 0.7961) compared to the control group (10.17 ± 0.7961). This increase was highly significant ($p < 0.0001$), confirming the successful induction of bradykinesia in the MPTP-induced PD model. Treatment with *B. clausii* (MPTP + *B. clausii*: 11.25 ± 0.7961) and *R. officinalis* (MPTP + *R. officinalis*: 9.833 ± 0.7961) significantly reduced the latency to descend in comparison to the MPTP- treated groups. The reductions in latency were 5.417 seconds (95% CI [3.153 to 7.680], $p < 0.0001$) and 6.833 seconds (95% CI [4.570 to 9.097], $p < 0.0001$), respectively, indicating that both treatments improved motor function. The combination of *B. clausii* and *R. officinalis* further improved bradykinesia in MPTP-treated mice (MPTP + *B. clausii* + *R. officinalis*: 13.40 ± 0.8349), showing a significant reduction in latency compared to the MPTP-treated groups (mean difference = 3.267 seconds, 95% CI [0.8926 to 5.641], $p = 0.0026$). This effect suggests a potential additive or synergistic impact of the combined treatment (Figure 4.2).

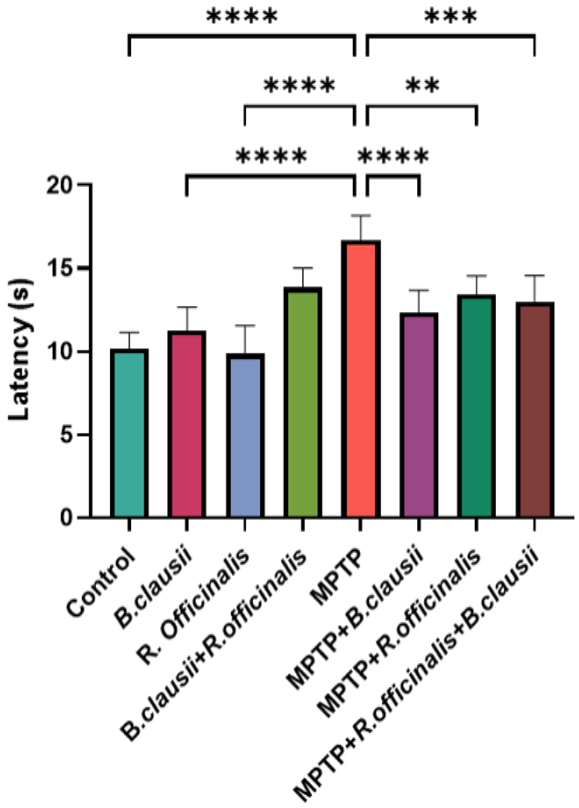


Figure 4.2 Effect of *R. officinalis* and *B. clausii* on bradykinesia assessed using pole test. Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni *post hoc test* to compare mean values with the standard error of the mean Mean ± SEM . The sample size was n=6, and significance levels were indicated as ****p<0.0001, ***p<0.001, and **p<0.01.

4.3 Effect of *R. officinalis* and *B. clausii* on motor dysfunction

The cylinder test was used to assess motor impairments, specifically increased forelimb asymmetry and reduced rearing behavior, in MPTP-induced PD mice. This test evaluates the ability of mice to explore their environment, and a reduction in rearing is indicative of motor dysfunction.

The forelimb asymmetry analysis revealed significant differences between treatment groups. MPTP administration caused a substantial increase in forelimb asymmetry compared to the control group ($p < 0.001$, 24.72 ± 1.92), confirming the motor deficits induced by this neurotoxin. The addition of *Bacillus clausii* to MPTP treatment significantly reduced asymmetry compared to MPTP alone ($p < 0.001$,13.50 ± 1.35). Similarly, the administration of *Rosmarinus officinalis* significantly improved motor performance compared to the MPTP group ($p < 0.001$,2.45 ± 1.67). The combination of *B. clausii* and *R. officinalis* further reduced forelimb asymmetry ($p < 0.001$, 4.21 ± 2.24), but this combination did not show significant improvement over individual treatments with *B. clausii* or *R. officinalis* ($p > 0.9999$,6.78 ± 0.45). Furthermore, significant differences were also observed between other groups, such as control vs. MPTP + *B. clausii* ($p = 0.001$,7.45 ± 1.34) and *R. officinalis* vs. MPTP + *R. officinalis* ($p = 0.0382$, 9.58 ± 1.22) (Figure 4.3A).

MPTP administration significantly reduced the number of rearing events, reflecting marked ($p < 0.0001$) motor deficits (MPTP-treated groups: 6.00 ± 0.9846) compared to the control group (13.00 ± 0.9846). Treatment with *B. clausii* (MPTP + *B. clausii*: 11.17 ± 0.9846) and *R. officinalis* (MPTP + *R. officinalis*: 10.67 ± 0.9846) significantly improved the number of rearing events ($p < 0.0001$) in comparison to the MPTP Treated Groups. The increase in rearing behavior for the *B. clausii* group was 5.167 events (95% CI [2.375 to 7.959], $p < 0.0001$), while for the *R. officinalis* group, the increase was

4.667 events (95% CI [1.875 to 7.459], $p < 0.0001$), demonstrating a significant restoration of motor activity. The combination of *B. clausii* and *R. officinalis* further enhanced the motor function (MPTP + *B. clausii* + *R. officinalis*: 10.67 ± 0.9846), with a mean increase of 3.00 rearing events compared to the MPTP Treated Groups (95% CI [0.2081 to 5.792], $p = 0.0286$). This improvement suggests a potential additive or synergistic effect of the combined treatment as shown in Figure 4.3B.

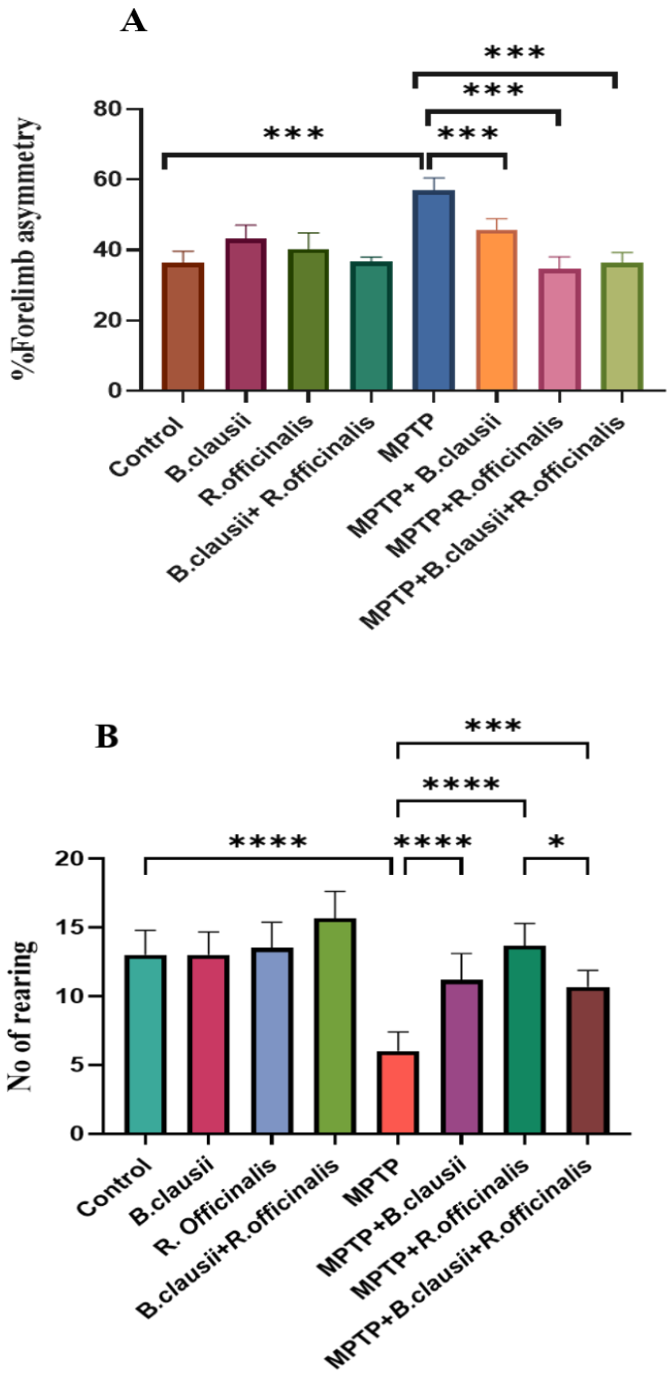


Figure 4.3 Effect of *R. officinalis* and *B. clausii* on Motor Function assessed through Cylinder Test

A) Forelimb asymmetry B) No of rearing events**.** Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni *post hoc test* to compare mean values with the standard error of the mean (SEM). The sample size was n=6, and significance levels were indicated as ****p<0.0001, ***p<0.001, and **p<0.01.

4.4 Histopathological Analysis of Neuronal Architecture

Histopathological examination of the hippocampal dentate gyrus (DG) region and sunstantia nigra was conducted across all experimental groups to assess any morphological changes following MPTP administration and treatment with *R. officinalis* and *B. clausii*.

The H&E stained sections revealed that MPTP administration resulted in pronounced neuronal loss and disorganization in the DG region of the hippocampus and substantia nigra. The MPTP-treated group showed a significant reduction in neuronal density (Mean = 8.750 ± 1.109, $p < 0.0001$) compared to the control group (Mean = 22.00 ± 0.4082), indicating severe cellular degeneration. This pathological alteration is indicative of the neurotoxic effects of MPTP, simulating neurodegeneration characteristic of PD. Treatment with *R. officinalis* (MPTP + *R. officinalis*: Mean = 23.75 ± 0.75, $p < 0.0001$) and *B. clausii* (MPTP + *B. clausii*: Mean = 22.50 ± 1.50, $p < 0.0001$) significantly restored neuronal architecture and density in the DG region, reversing the detrimental effects of MPTP. Notably, the combination treatment (MPTP + *R. officinalis* + *B. clausii*) exhibited a near-complete recovery in neuronal structure, highlighting the potential synergistic effect of these two compounds in promoting neuroprotection.

The histological analysis further reveals that the neuroprotective effects of *R. officinalis* and *B. clausii* are comparable to or even greater than the control group in some cases, with significant preservation of neuronal density and architecture at 10X magnification. Cell counting confirmed these results, demonstrating substantial improvements in the dentate gyrus following the combined treatment, suggesting a potent reversal of MPTP-induced neurodegeneration (Figure 4.4).

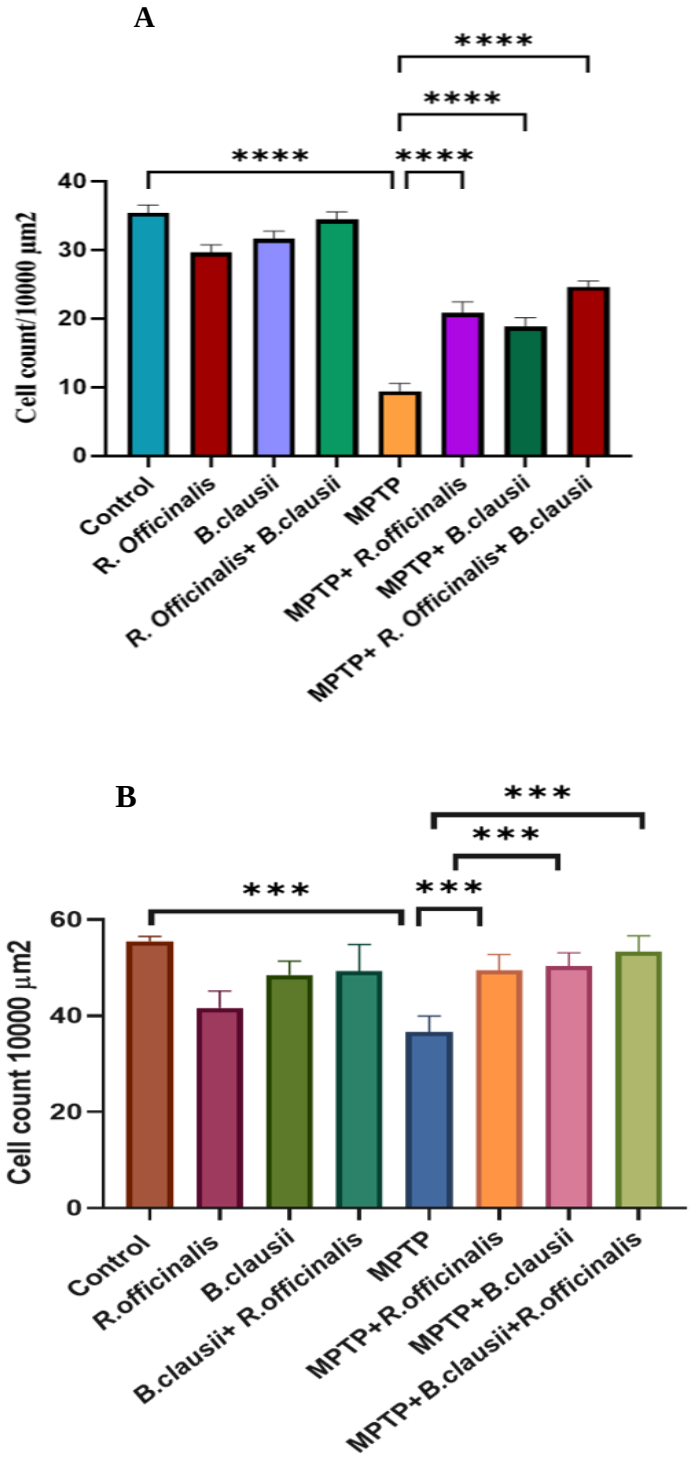
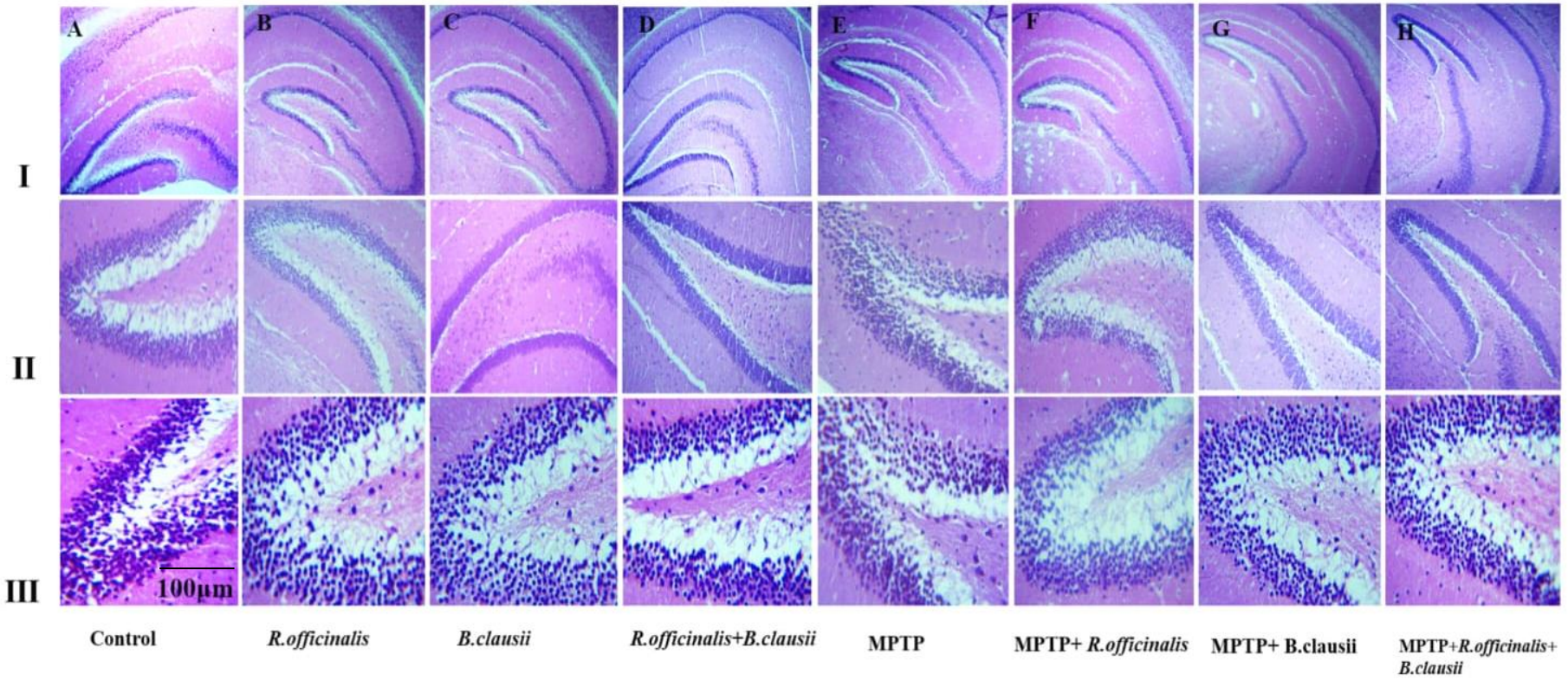


Figure 4.4 Effect of *Bacillus clausii* and *Rosmarinus officinalis* on neuronal density od DG region of hippocampus (A) and substantia nigra (B). Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni *post hoc test* to compare mean values with the standard error of the mean (Mean ± SEM SEM). The sample size was n=6, and significance levels were indicated as ****p<0.0001, ***p<0.001, and **p<0.01.

4.4.1 Histopathological Analysis of the Dentate Gyrus regions of hippocampus

The histopathological examination of the DG regions of hippocampus revealed significant neurodegeneration in the MPTP-treated group, marked by disrupted neuronal architecture. In contrast, treatment with *R. officinalis* and *B. clausii* showed substantial neuroprotective effects, with both groups demonstrating improved cellular organization and reduced structural damage compared to MPTP-treated group. The combined treatment of *R. officinalis* and *B. clausii* provided the most pronounced protection, with near-normal hippocampal structure and minimal signs of degeneration, suggesting a potential synergistic effect. (Figure 4.5)



Figures 4.5. H & E-stained coronal sections of DG regions. A) Control B) *R. Officinalis* C) *B.clausii* D) *R. Officinalis* and *B.clausii E)* MPTP F) MPTP+ *R.officinalis* G) MPTP + *B.clauii* H) MPTP + *R.officinalis* + *B.clausii*. Magnification I (4X), II (10X), III (40X).

4.4.2 Histopathological Analysis of the Substantia Nigra Pars Compacta

The histopathological examination of the SNpc revealed significant neurodegeneration in the MPTP-treated group, marked by disrupted neuronal architecture. In contrast, treatment with *R. officinalis* and *B. clausii* showed substantial neuroprotective effects, with both groups demonstrating improved cellular organization and reduced structural damage compared to MPTP-treated group. The combined treatment of *R. officinalis* and *B. clausii* provided the most pronounced protection, with near-normal SNpc structure and minimal signs of degeneration, suggesting a potential synergistic effect.

(Figure 4.6)

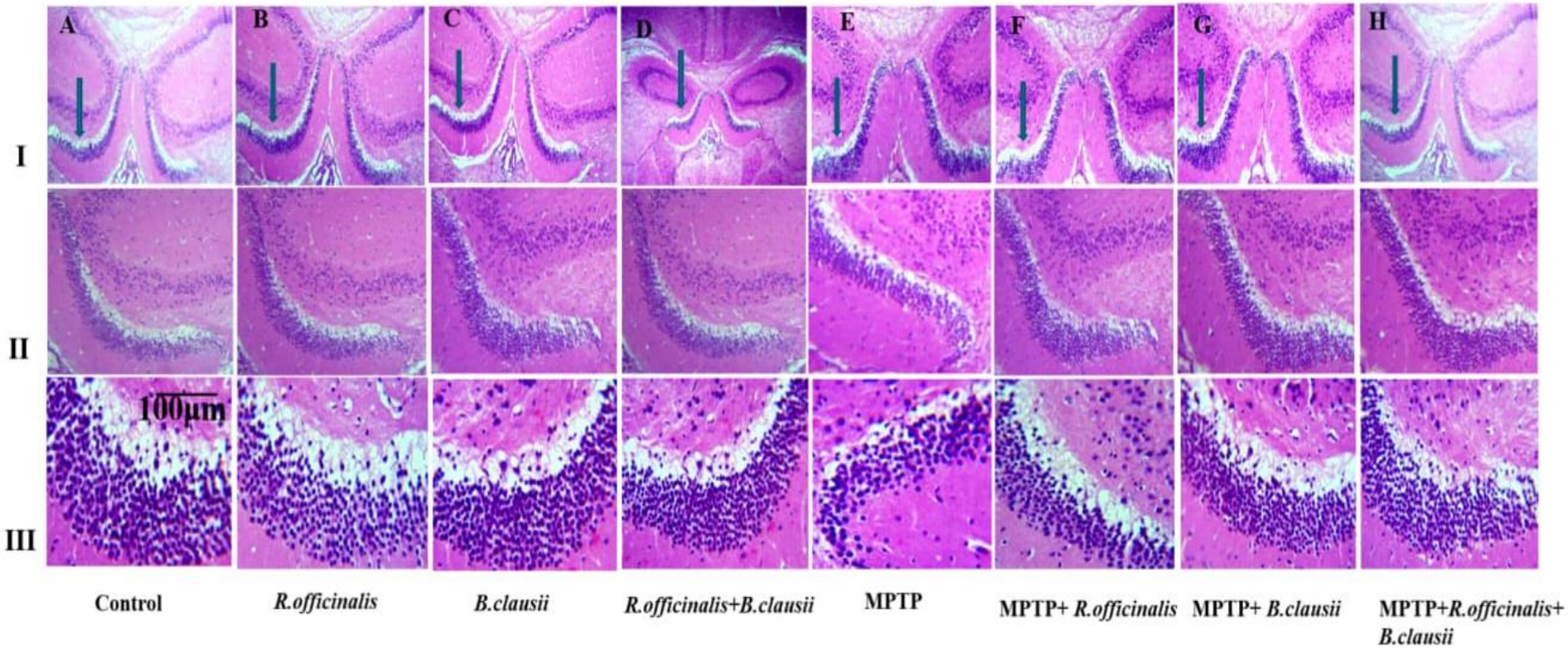


Figure 4.6. H & E-stained coronal sections of SNpc, here I is 4X , II is 10X and III is 40X. A) Control B) *R. Officinalis* C) *B.clausii* D) *R. Officinalis* and *B.clausii E)* MPTP F) MPTP+ *R.officinalis* G) MPTP + *B.clauii* H) MPTP + *R.officinalis* + *B.clausii*

4.5 Gene expression analysis

4.5.1 Effect of *B. clausii* and *R. officinalis* on IL-6 Expression

In hippocampus, the MPTP treated groups exhibited markedly higher IL-6 expression (3.473 ± 0.07724, $p<0.001$) compared to the control (1.000 ± 0.07724). The *B. clausii* ($p<0.001$, 1.639 ± 0.07724), *R. officinalis* (1.704 ± 0.07724), and the combination treatment i.e. *B. clausii* + *R. officinalis* ($p<0.001$, 1.476 ± 0.07724) showed significantly lower IL-6 levels post MPTP treatemnet as compared to the the MPTP-treated group (Figure 4.7A).

Similarly, In substantia nigra, the MPTP-treated groups showed a substantial increase in IL-6 expression (2.465 ± 0.06831, $p<0.001$) compared to the control group (1.000 ± 0.06831). MPTP-treated groups with *B. clausii* (0.7169 ± 0.06831, $p<0.001$), *R. officinalis* (0.8025 ± 0.06831, $p<0.001$), and the combination treatment of *B. clausii* + *R. officinalis* (1.295 ± 0.06831, $p<0.001$) significantly reduced the IL-6 expression compared to MPTP-treated group (Figure 4.7B).

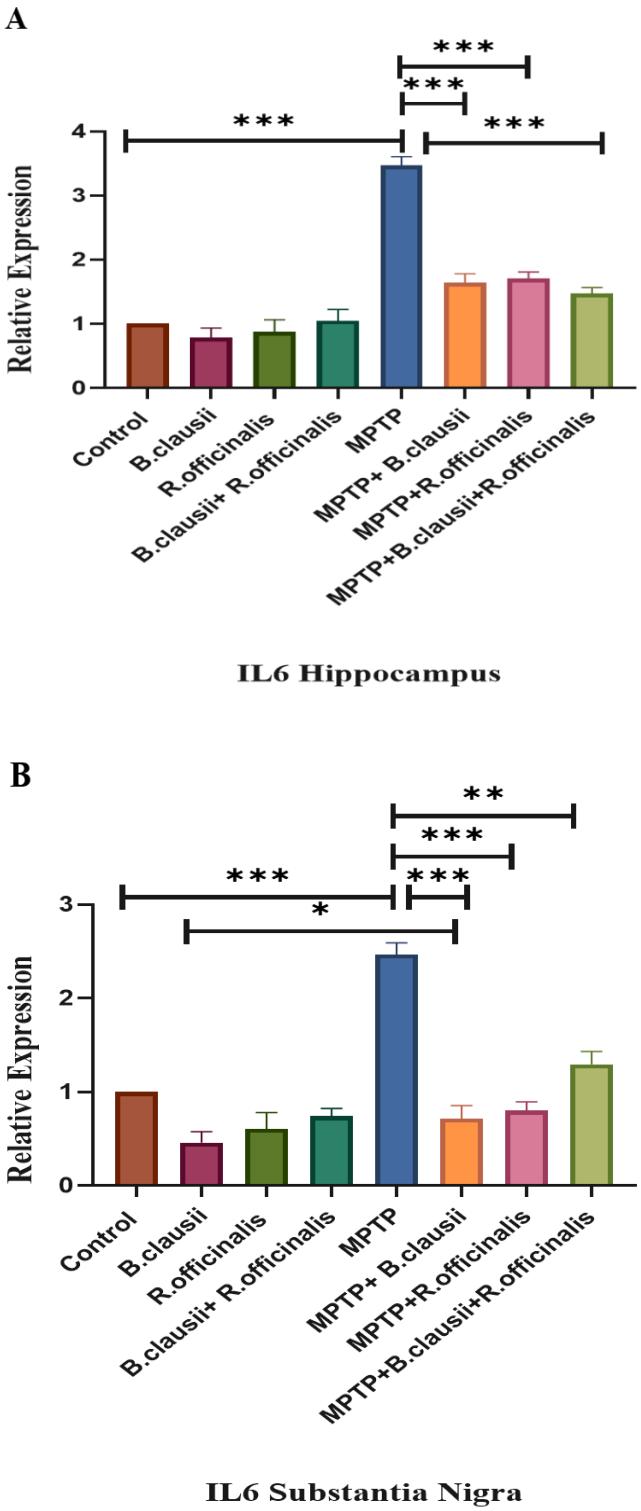


Figure 4.7 Effect of *B. clausii* and *R. officinalis* on IL-6 Expression in the Hipocampus (A) and substantia nigra (B) : Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni test to compare mean values with the standard error of the mean (SEM). The sample size was n=6, and significance levels were indicated as ****p<0.0001, ***p<0.001, and **p<0.01.

4.5.2 Effect of B. clausii and R. officinalis on TNF-α Expression

In hippocampus, the MPTP -treated group displayed a significant increase in TNF-α levels (2.709 ± 0.07919, $p<0.001$) relative to the control group (1.000 ± 0.07919). MPTP-treated groups followed with *B. clausii* (1.605 ± 0.07919, $p<0.001$), *R. officinalis* (1.487 ± 0.07919, $p<0.001$), and combination treatment (1.141 ± 0.07919, $p<0.001$) effectively reduced TNF-α expression compared to the MPTP-treated group (Figure 4.8A).

In the substantia nigra the MPTP-treated group exhibited a substantial increase in TNF-α expression (2.805 ± 0.05418, $p<0.001$) when compared to the control group (1.000 ± 0.05418). MPTP-treated groups followed with *B. clausii* (1.172 ± 0.05418, $p<0.001$), *R. officinalis* (1.773 ± 0.05418, $p<0.001$), and combination treatment i.e. *B. clausii* + *R. officinalis* (1.709 ± 0.05418, $p<0.001$) showed significantly reduced TNF-α expression levels ($p<0.001$) relative to MPTP-treated group(Figure 4.8B).

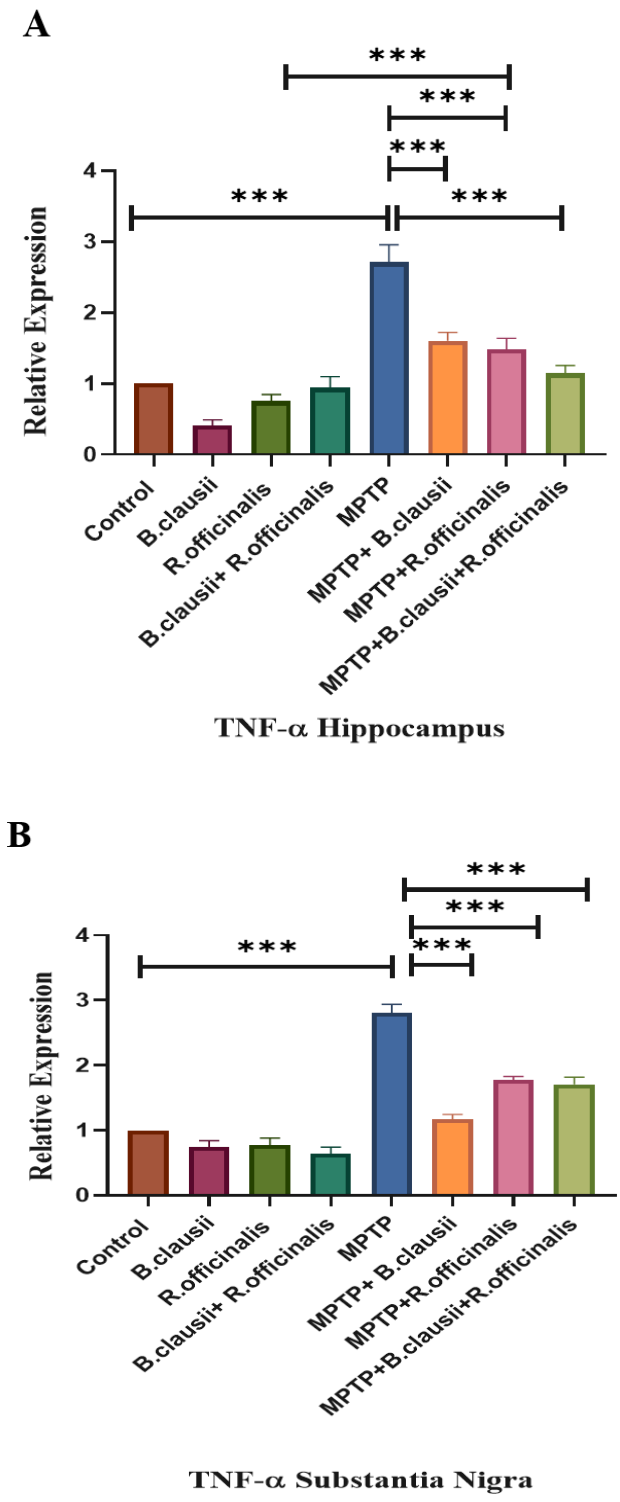


Figure 4.8 Effect of *B. clausii* and *R. officinalis* on TNF-α Expression in the Hipocampus (A) and Substantia Nigra (B): Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni *post hoc test* to compare mean values with the standard error of the mean (SEM). The sample size was n=6, and significance levels were indicated as ****p<0.0001, ***p<0.001, and **p<0.01.

4.5.3 Effect of *B. clausii* and *R. officinalis* on SOD1 Expression

In hippocampus, the MPTP-treated groups exhibited a marked decrease in SOD1 expression (0.2313 ± 0.05916, $p<0.001$) as compared to the control group (1.000 ± 0.05916). However, treatment with *B. clausii* (1.449 ± 0.05916, $p<0.001$), *R. officinalis* (2.114 ± 0.05916, $p<0.001$), and their combination (1.703 ± 0.05916, $p<0.001$) post MPTP -exposure, significantly reduce the SOD1 levels ascompared to the MPTP-treated group(Figure 4.9A).

The expression of SOD1 in the substantia nigra in the MPTP treated groups showed a notable reduction (0.1171 ± 0.05572, $p<0.001$) as compared to the control group (1.000 ± 0.05572). -Treatments with *B. clausii* (0.9099 ± 0.05572, $p<0.01$), *R. officinalis* (2.406 ± 0.05572, $p<0.001$), and the combination of these i.e. *B. clausii* + *R. officinalis* (1.792 ± 0.05572, $p<0.001$) induce significant increase in the expression relative to MPTP-treated group(Figure 4.9B).

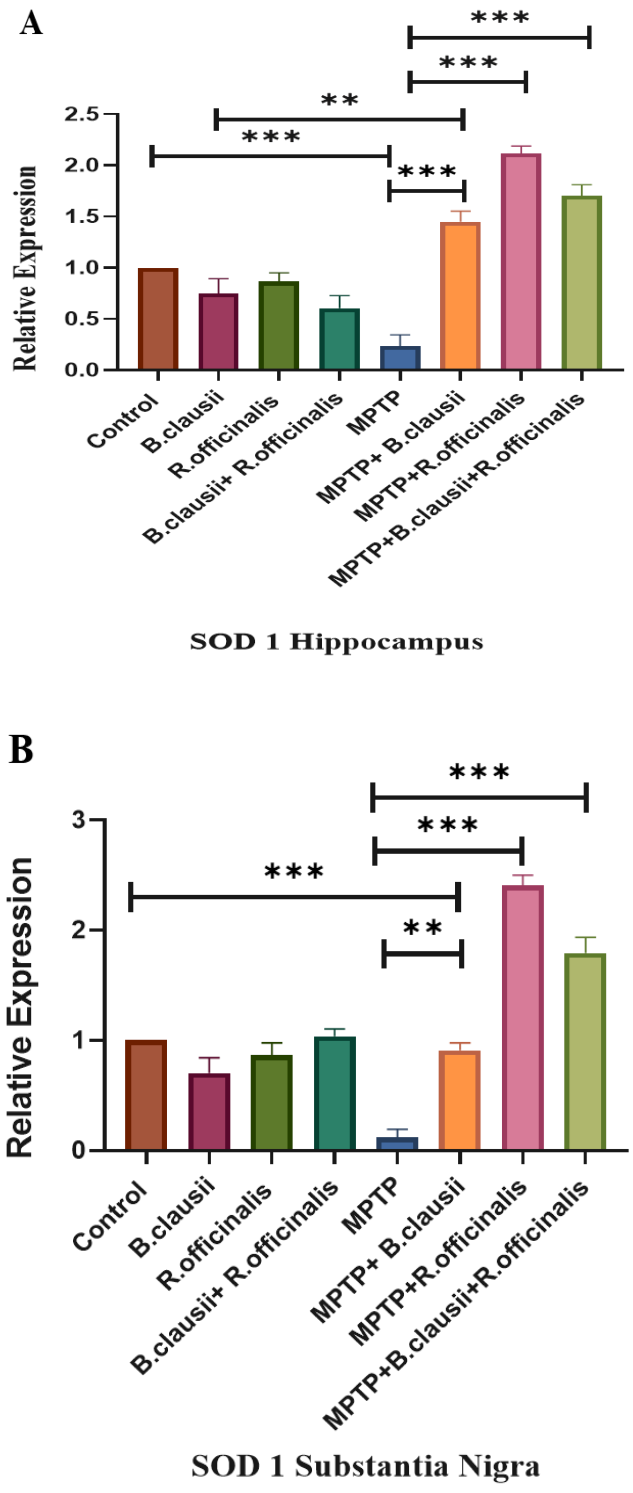


Figure 4.9 Effect of B. clausii and R. officinalis on SOD-1 Expression in the Hippocampus (A) and Substantia Nigra (B) : Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni *post hoc test* to compare mean values with the standard error of the mean (SEM). The sample size was n=6, and significance levels were indicated as ****p<0.0001, ***p<0.001, and **p<0.01.

4.5.4 Effect of *B. clausii* and *R. officinalis* on SOD2 Expression

The expression of SOD2 in the hippocampus in the MPTP treated groups exhibited a pronounced reduction in SOD2 expression (0.2522 ± 0.05332, $p<0.001$) relative to the control group (1.000 ± 0.05332). Treatments with *B. clausii* (1.548 ± 0.05332, $p<0.001$), *R. officinalis* (1.822 ± 0.05332, $p<0.001$), and their combination i.e. *B. clausii* + *R. officinalis* (2.760 ± 0.05332, $p<0.001$) post MPTP exposure showed a significant improvemnet in SOD2 expression compared to the MPTP-treated groups(Figure 4.10A).

The expression of SOD2 in the substantia nigra was also significantly affected in the disease group. Similar expression dysregulation was observed in the MPTPtreatment groups that showed a marked reduction in SOD2 expression (0.5317 ± 0.05604, $p<0.001$) compared to the control group (1.000 ± 0.05604). Treatments with *B. clausii* (1.752 ± 0.05604), *R. officinalis* (2.060 ± 0.05604), and their combination i.e. *B. clausii* + *R. officinalis* (3.760 ± 0.05604) post MPTP exposureimproved the SOD2 expression in comparision to the MPTP-treated group. The combination of *B. clausii* and *R. officinalis* showed the highest SOD2 expression level, suggesting a significantt antioxidative response (Figure 4.10B).

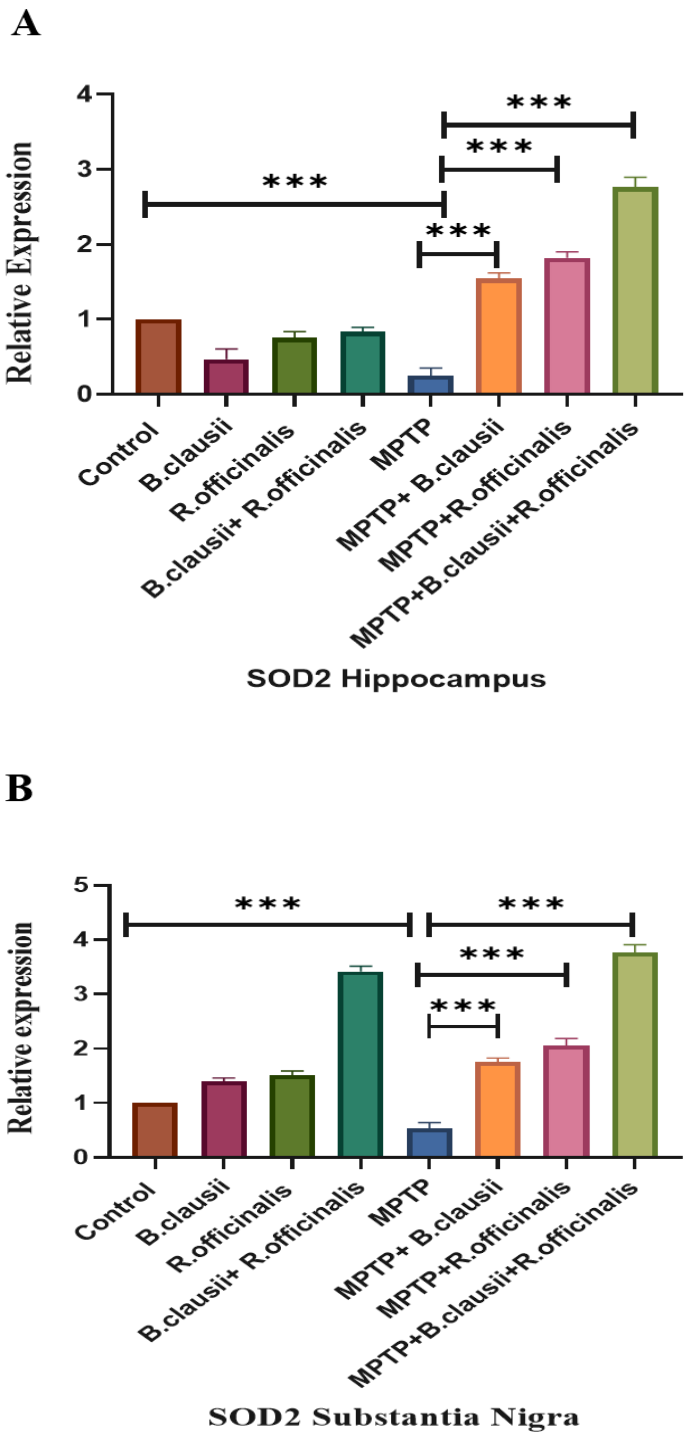


Figure 4.10 Effect of B. clausii and R. officinalis on SOD-2 Expression in the Hippocampus (A) and Substantia Nigra (B): Statistical analysis was performed using GraphPad Prism, applying one-way ANOVA followed by the Bonferroni *post hoc test* to compare mean values with the standard error of the mean (SEM). The sample size was n=6, and significance levels were indicated as ****p<0.0001, ***p<0.001, and **p<0.01.

4.6 In-Silico analysis

4.6.1 Molecular docking studies of Bioactive compounds of *R.officinalis*

The geometrics of the receptor–ligand interaction for the chosen compounds were predicted by employing molecular docking. The docking scores of the bioactive compounds of *R.officinalis* with interacting residues SNCA, APOE, PRKN, IL-6, TNF-α SOD1, SOD2 including H bonds and van der Waals interacting residues are described in Table 4.1. The results are shown for RA, CA, Carnosol, Limonene, camphene, genkwanin and UA with the PD target molecules as the potency depends on the binding energy interaction and the H bonding. (Table 4.1) and their respective 2D and 3D structures are presented in Figure 4.11, 4.12, 4.13 4.14, 4.15, 4.16, 4.17, visualized by Biovia discovery studio.

Table 4.1 Docking interaction of R. officinalis active compounds with the target proteins.

Target	Ligand	Binding energy	Interacting residues
SNCA	Ursolic acid	-7.0	Van der Waals; Lys 12, Val 15, Ala19, Thr22, al 26 Alkyl: Val 16, Lys 23
SNCA	Carnosic acid	-6.4	Van der Waals: Asp2 Pi-Pi: Phe4 Alkyl-Pi alkyl: Leu8, Met5
SNCA	Carnosol	-7.0	H Bond: Phe94 Van der Waals: Val 95, Lys97, Lys96, gly93, Thr92
SNCA	Rosmarinic acid	-6.1	H bond: Asp 119, Glu123 Pi alkyl: Ala 124 Van der Waals: Pro 117, Val 118, Tyr 125
SNCA	Genkwanin	-5.8	H bonds: Ser9 Van der Waal: Lys 12

			Pi-anion: Glu 13 Pi alkyl: Ala 17, Val 16
SNCA	Limonene	-4.4	Van der Waals: Gln 62, gly67 Alkyl: Val66, Val63, Val70
SNCA	Camphene	-4.0	Van der Waals: Glu114, Met116, Asp115 Alkyl: Pro117, Leu113
SNCA	Levodopa	-4.4	H bond: Glu13, Ser9 Van der Waals: Ala17 Amide Pi-stacked: Lys 12 Pi alkyl: Val 16
APOE	Ursolic acid	-7.1	Van der Waals: Ala 62, Glu59, Arg 32, Asp35 Pi-alkyl: Leu 63, Tyr 36, Trp39
APOE	Carnosic acid	-6.8	H Bond: Gln 24 Van der Waals: Glu 77, Trp26, Ala 73, Glu70 Alkyl: Tyr 74, Arg 25
APOE	Carnosol	-6.8	Van der Waals: Glu 77, Ala 73, Glu 70, Trp 26, Gln 24 Alkyl: Arg 25, Tyr 74

APOE	Rosmarinic acid	-6.0	H bond: Glu59, Glu50 Pi-Pi stacked: Tyr36, Trp39 Van der Waals: Leu63, Asp35, Arg32, Gln55, Gln46
APOE	Genkwanin	-6.5	H bonds: Arg32 Van der Waals: Asp 35, Gln 46, Glu 50, Glu 59 Alkyl: Leu 63 Pi- pi t shaped: Tyr 36, Trp 39
APOE	Limonene	-4.8	Alkyl: Trp26, Ala 73, Arg25, Tyr74 Van der Waals: Glu77, Glu70, Gln24
APOE	Camphene	-4.8	Van der Waals: Gln 24, Glu 70, Glu77 Alkyl: Arg 25: Pi alkyl: Tyr 74
APOE	Levodopa	-5.2	H bond: Glu77, Glu70, Trp26 Pi-sigma: Arg25 Van der Waal: Tyr74, Gln24, Leu71, Ala73,
PRKN	Ursolic acid	-7.1	Alkyl: Arg6, Asn8, Arg 8, Arg42, Leu41, Val36, Gln40 C-H bond: Pro37

PRKN	Carnosic acid	-5.9	H bond: Arg 6 Alkyl: Val 43, Val5, Leu 61 Van der Waals: Ala 46, Ile44, Leu50, Phe4, Gln64, Phe45, Leu26
PRKN	Carnosol	-6.0	Alkyl: Arg6, Phe4, Val43, Val 5, Leu61, Phe45, Van der Waals: Ala46. Gln64, Ile44, Leu50, Leu26
PRKN	Rosmarinic acid	-5.6	H bond: Arg 42 Alkyl: Leu61 C-H bond: Val5, Ile44, Val43, Van der Waals: Phe45, Leu50, leu26, Lys27, Val30, Phe4, Gln64, Phe7
PRKN	Genkwanin	-6.4	H bond: Arg 6, Lys27 Alkyl: Leu26, Val43, Leu61, Val5 Van der Waals: Ile44, Val30, Ile23, Leu50, Phe45, Phe4
PRKN	Limonene	-4.5	Alkyl: Leu61, Val43, Leu26, Val30, Ile23, Phe45, val5

PRKN	Camphene	-4.1	Alkyl: Val5, Leu26, Val30, Val43, Leu61, Ile23 Van der Waals: Val 3, Ile44, Phe45.
PRKN	Levodopa	-4.7	H bond: Ile23, Ile44 Alkyl: Leu26, Val43 C-H bond: leu61 Van der Waals: Lys27, Val30, Arg6, Val5, Phe4, Val3
IL-6	Ursolic acid	-7.9	Van der Waals: Glu 100, Asn 145, Pro 140, Glu 94, Val 97, Leu 93, Thr 139, Glu 96, Ile 124, Lys 121, Gln 125 C-H Bond: Pro 142
IL-6	Carnosic acid	-7.1	Van der Waals: Leu 65, Arg 169, Phe 95, Phe 174, Ser 177, Met 68, Phe 75, Ala 69, Asn 62 H bond: Ser 170 Pi- anion: Glu 173 Alkyl: Pro 66, Leu 166
IL-6	Carnosol	-7.2	H Bond: Arg 169 Van der Waals: Asn 62, Leu 65, Ser 170, Phe 174, Met 68, Ala 69, Phe 75, Ser 177

			Pi-anion: Glu 173 Alkyl: Leu 166, Pro 66
IL-6	Rosmarinic acid	-6.3	H bond: Lys 121, Asn 145 Pi alkyl: Leu 93 Van der Waals: Ile 124, Glu 96, Val 97, Glu 94, Pro 140, Asp 141, Thr 139, Ala 146 C-H bond: Pro 142
IL-6	Genkwanin	-6.8	H bonds: Asn 62, Leu 63 Van der Waal: Leu 65, Ser 170, Phe 174, Phe 75, Arg 169, Ala 59 C-H bond: Pro 66 Pi-anion: Glu 173 Pi alkyl: Leu 166, Met 68
IL-6	Limonene	-5.1	Van der Waals: Glu 43, Thr 44, Ser 108, Glu 107, Arg 105 Alkyl: Phe 106
IL-6	Camphene	-4.7	Van der Waals: Glu114, Met116, Asp115 Alkyl: Pro117, Leu113

IL-6	Levodopa	-5.7	H bond: Glu173 Van der Waals: Leu 63, Asn 64, Leu 65, Pro 66, Leu 166, Lys 67, Met 68, Phe 174, Arg 169 C-H bond: Asn 162 Donor- Donor: Ser 170
TNF-α	Ursolic acid	-7.5	Van der Waals: Cys B: 101, Pro B: 100, Gln A: 102, Glu B: 104, Arg A: 103, Arg B: 103, Pro C: 100, Cys C 101, Gln C: 102, Arg C : 103, Glu C: 104, Glu A: 104 H Bond: Gln B: 102
TNF-α	Carnosic acid	-6.3	H Bond: Leu A: 26, Asn A: 46
TNF-α	Carnosol	-6.5	Van der Waals: Leu B: 143, Gln B: 21, Glu B: 23, Ala B: 22, Gly B: 24, Leu B: 142, Pro B: 139, Asp B: 140 H Bond: Lys B: 65, Pro B: 20 Pi Sigma: Phe B: 144
TNF-α	Rosmarinic acid	-9.1	H bond: Ser C: 99, Ser B: 99, Glu B: 116, Gln B: 102, Tyr A: 115, Pi-anion: Glu C: 116

			Van der Waals: Lys C: 98, Glu A: 116, Pro C: 100, Trp A: 114, Pro A: 100, Gln C: 102, Pro B: 100, Gln A: 102, Ser A: 99, Tyr B: 115, Glu B: 104, Arg B: 103, Cys B: 101 Pi-anion: Glu C: 116
TNF-α	Genkwanin	-8.6	H bonds: Arg B: 103, Gln B: 102 Van der Waals: Cys B: 101, Pro B: 100, Gln A: 102, Glu B: 116, Glu B 104, Pro A: 100, Gln C: 102, Cys A: 101, Ser C: 99, Glu A: 116, Glu B: 116 Alkyl: Lys B: 98 C H bond: Tyr C: 115, Glu C: 116, Ser B: 99
TNF-α	Limonene	-4.9	Alkyl: Arg B: 103 Van der Waals: Gln A: 102, Gln B: 102, Gln C: 102, Glu B: 104, Glu C: 104, Cys A: 101
TNF-α	Camphene	-6.3	Van der Waals: Tyr A: 119, Tyr B:119, Tyr C: 119, Ile A: 118, Ile B: 118, Ile C: 118, Pro a: 117, Pro C: 117, Lus A98, Lys B:98, Lys C: 98 Alkyl: Pro 117
TNF-α	Levodopa	-6.9	H bond: Tyr C:119, Lys C: 98, Tyr A: 119 Pi-alkyl: Ala A: 96, Pro B: 117

			Donor-Donor: Gln B: 61 Van der Waal: Tyr B: 119, Lys B: 98, Ile B: 118, Gln C: 61, Pro C: 117, Ile C: 118, Pro A: 117, Lys A: 98, Ile A: 118
SOD-1	Ursolic acid	-6.0	Van der wall: Lys 76, Val 74, Gln 19, Ala 73, Ala 17, Gln 49, Asp 48 C-H bond: Val 18
SOD-1	Carnosic acid	-5.3	Van der Waals: Lys 76, Val 74, Gln 19, Ala 73, Ala 17, Gln 49, Asp 48 C-H Bond: Val 18
SOD-1	Carnosol	-5.8	H- Bond: Val 18, Met 20 Van der waal: Ala 73, Gln 49, Thr 21 Alkyl: Val 74, Ala 17 Van der Waals: Ala46. Gln64, Ile44, Leu50, Leu26
SOD-1	Rosmarinic acid	-5.2	H bond: Gln 49, Val 18, Ala 17 Van der Waals: Asp 48, Val 74, ala 73
SOD-1	Genkwanin	-5.4	Alkyl: Val 42 Pi-sigma: Gln 34 Van der Waals: Asp 41, Val 39, Gly 35, Val 36

SOD-1	Limonene	-3.7	Alkyl: Val 42 Van der Wall: Gln 34, Asp 41, Val 39, Gly 35, Gln 40
SOD-1	Camphene	-3.6	Alkyl: Val 74, Ala 17 Van der Wall: Lys 76, Val 18, Gln 19, Ala 73
SOD-1	Levodopa	-4.5	H bond: Arg 30, Asp 41, Val 42, Gly 35, Val 39 Van der Waals: Glu 43, Leu 33, Val 36, Gln 40 Pi-sigma: Gln: 34
SOD-2	Ursolic acid	-10.5	H bond: His 171, Glu 170 Van der Wall: Phe 124, Tyr 173, Arg 181, Asn 179, Ala 121, Ser 122
SOD-2	Carnosic acid	-9.0	H bond: Arg 123 Van der Waal: Ser 122, Ala 121, Gly 125, Glu 170, Asn 179, Tyr 173 C-H bond: Phe 124
SOD-2	Carnosol	-8.7	H Bond: Asn 74 C-H Bond: Ala 121 Pi Pi T-shaped: Phe C:34, Phe D: 124

SOD-2	Rosmarinic acid	-7.6	H Bond: His 30, Ala 121, Arg 123 Van der Waal: Asn 37, Phe 34, Trp 169, His 171 Pi Pi t shaped: Phe 124 Pi Alkyl: Pro 182
SOD-2	Genkwanin	-8.3	H Bond: Asp 136, Lys 134, Gly 160, Van der Waal: Ser 159, Thr 58, Phe 161, Gly 135 Alkyl: Lys 204, Leu 150 Pi-sigma: Leu 133
SOD-2	Limonene	-5.3	Van der Waal: Thr 33, Phe 34, Trp 169, His 171, His 30 Pi-alkyl: Phe 124
SOD-2	Camphene	-5.9	Van der waal: Asn 37, Thr 33, Phe 3, His 30, His 171 Pi-alkyl: Phe 124, Trp 169
SOD-2	Levodopa	-6.1	H Bond: Ala 121, His 30, Glu 170 Van der waal: Gly 125, Phe 124, Arg 123, Ser 122, Arg181,

4.6.1.1 UA and carnosol exhibit strong binding interaction with SNCA

The binding interactions of all the bioactive compounds of *R.officinalis* revealed that out of all the compounds UA and carnosol (both have -7.0 binding energy) has the highest binding potential with SNCA forming different interactions with the residues of SNCA. Whereas, CA and RA had moderate binding energies i.e. -6.4 and -6.1 making Van der Waals and other interactions with SNCA. Levodopa, which is therapeutic agent for PD had the weakest binding energy of -4.4 compared to more potent ligands like UA and carnosol. (Fig 4.11A-B)

4.6.1.2 UA exhibits strong binding interactions with APOE

UA showed the highest binding affinity with binding energy of -7.1 making different interactions with residues of APOE. CA and carnosol had the same binding energies of -6.8 showing the same van der Waal interactions (Glu 77, Trp26, Ala 73, Glu70) and alkyl interactions (Tyr 74, Arg 25) with APOE, whereas the levodopa had -5.8 binding energy showing poor interactions with APOE as compared to other ligands. (Figure 4.12A-B)

4.6.1.3 UA exhibits strong binding interactions with PRKN

UA showed the highest binding affinity with PRKN also, having binding energy of -7.1 and forming different interactions. Genkwanin with binding energy of -6.4, can also be considered as a good interaction showing good affinity and making van der Waals, H-bond and alkyl interactions with PRKN. Levodopa had binding energy of -4.7 showing that it had the weakest binding potential than other compounds with PRKN. (Figure 4.13A-B)

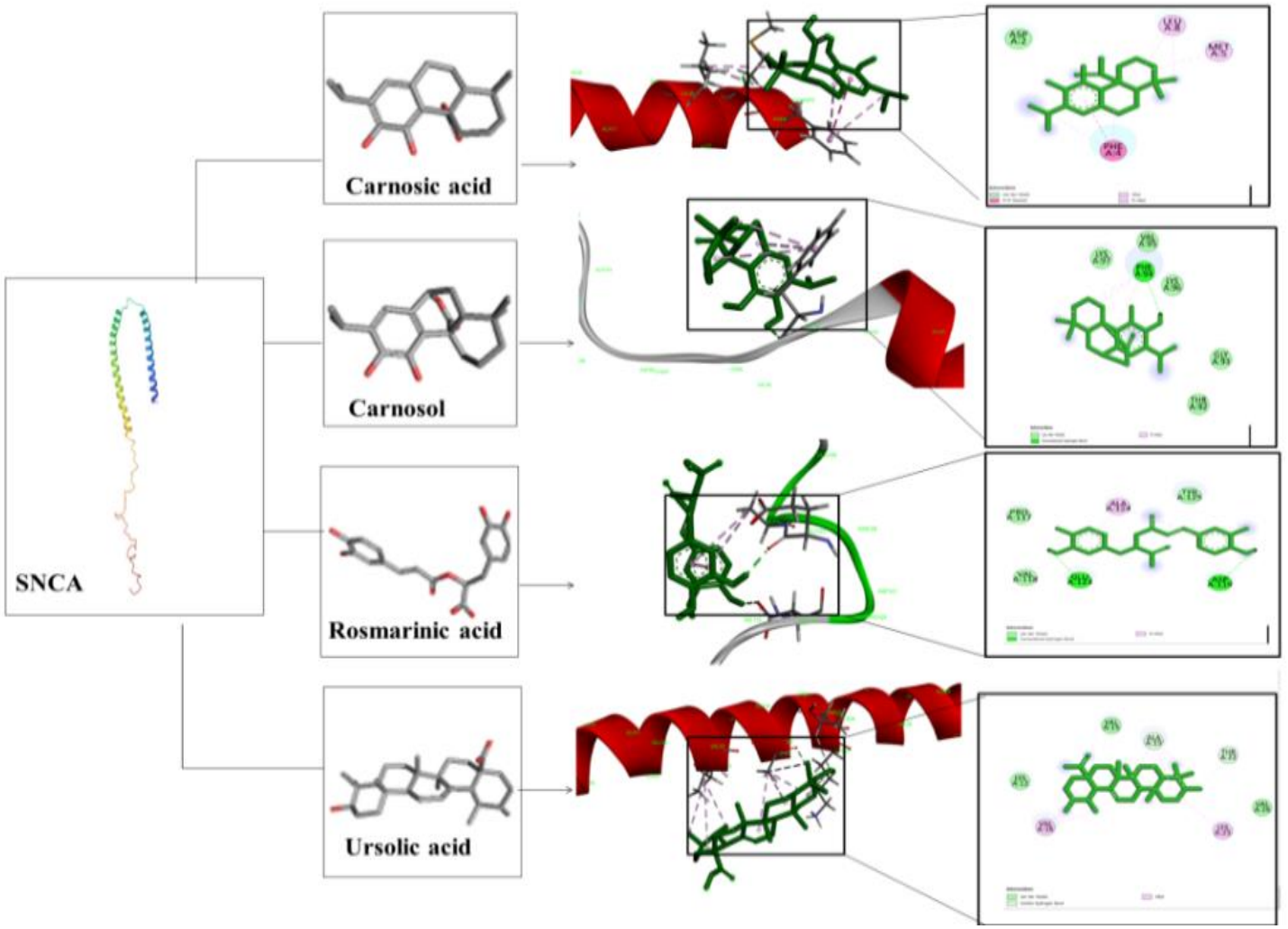


Fig 4.11A: 2D and 3D interactions of SNCA (PDB ID: 1xq8) with Bioactive compounds of *R.officinalis*

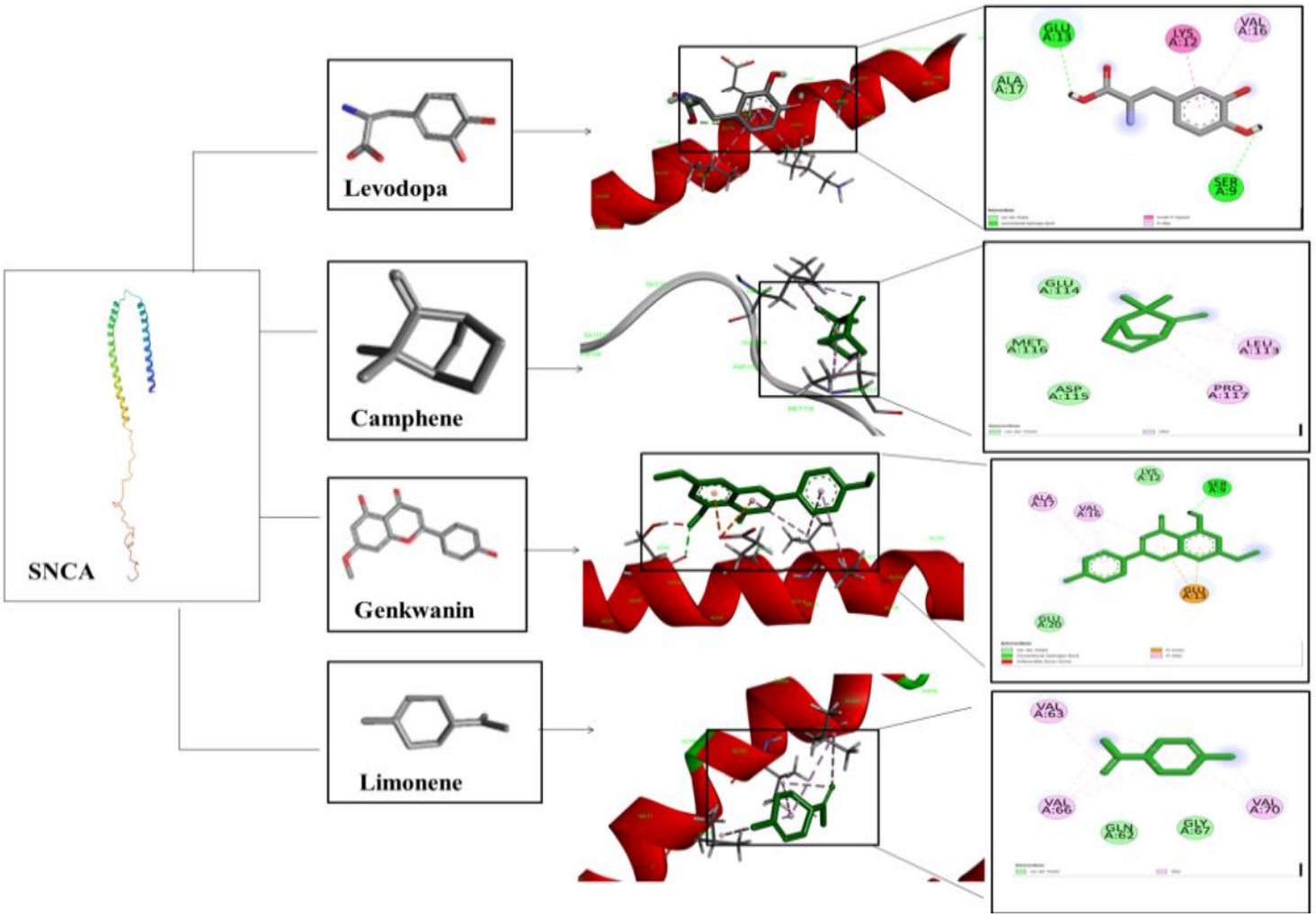


Fig 4.11B: 2D and 3D interactions of SNCA (PDB ID: 1xq8) with Bioactive compounds of *R.officinalis*

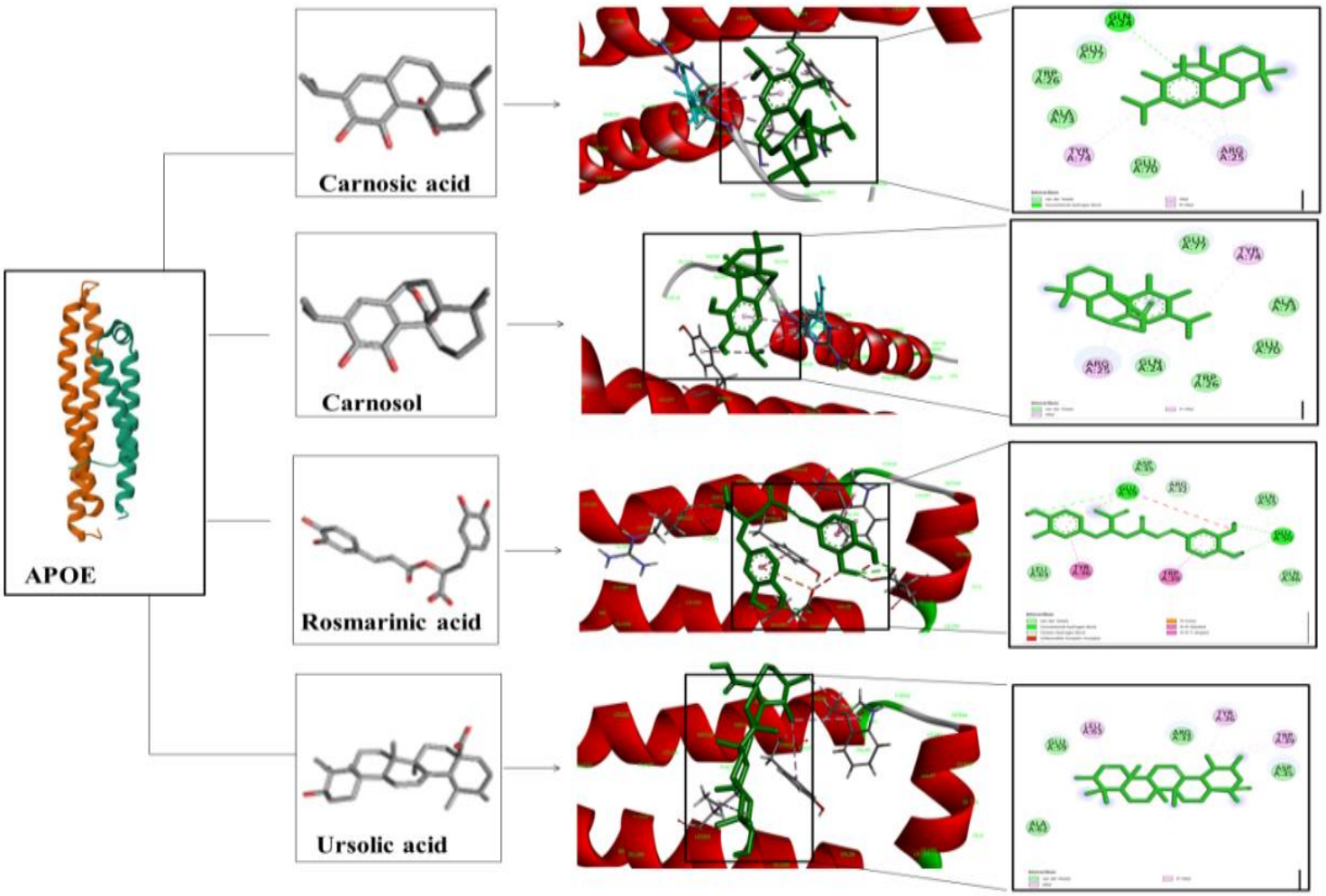


Fig 4.12A 2D and 3D interactions of APOE (PDB ID: 6v7m) with Bioactive compounds of *R.officinalis*

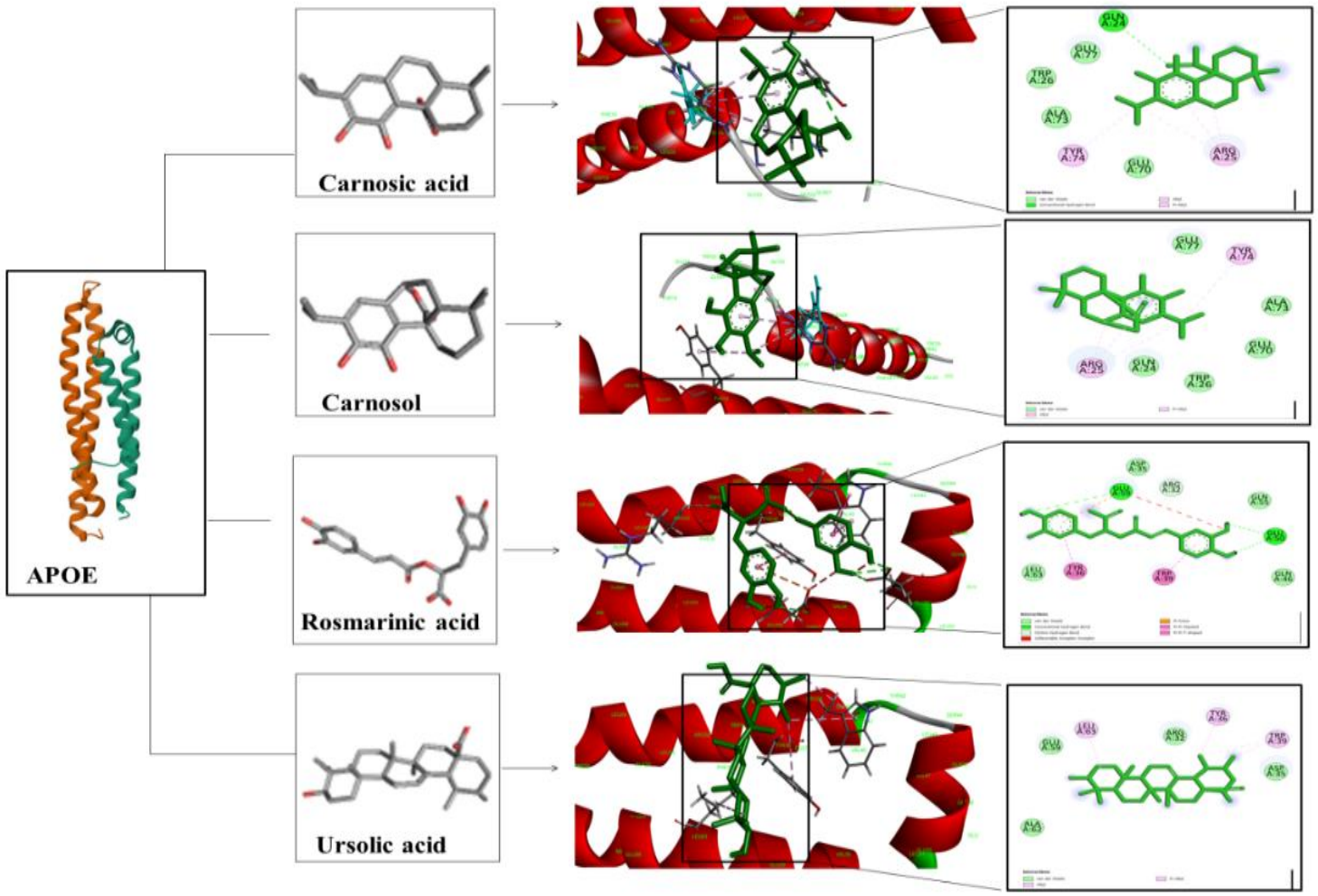


Fig 4.12B 2D and 3D interactions of APOE (PDB ID: 6v7m) with Bioactive compounds of *R.officinalis*

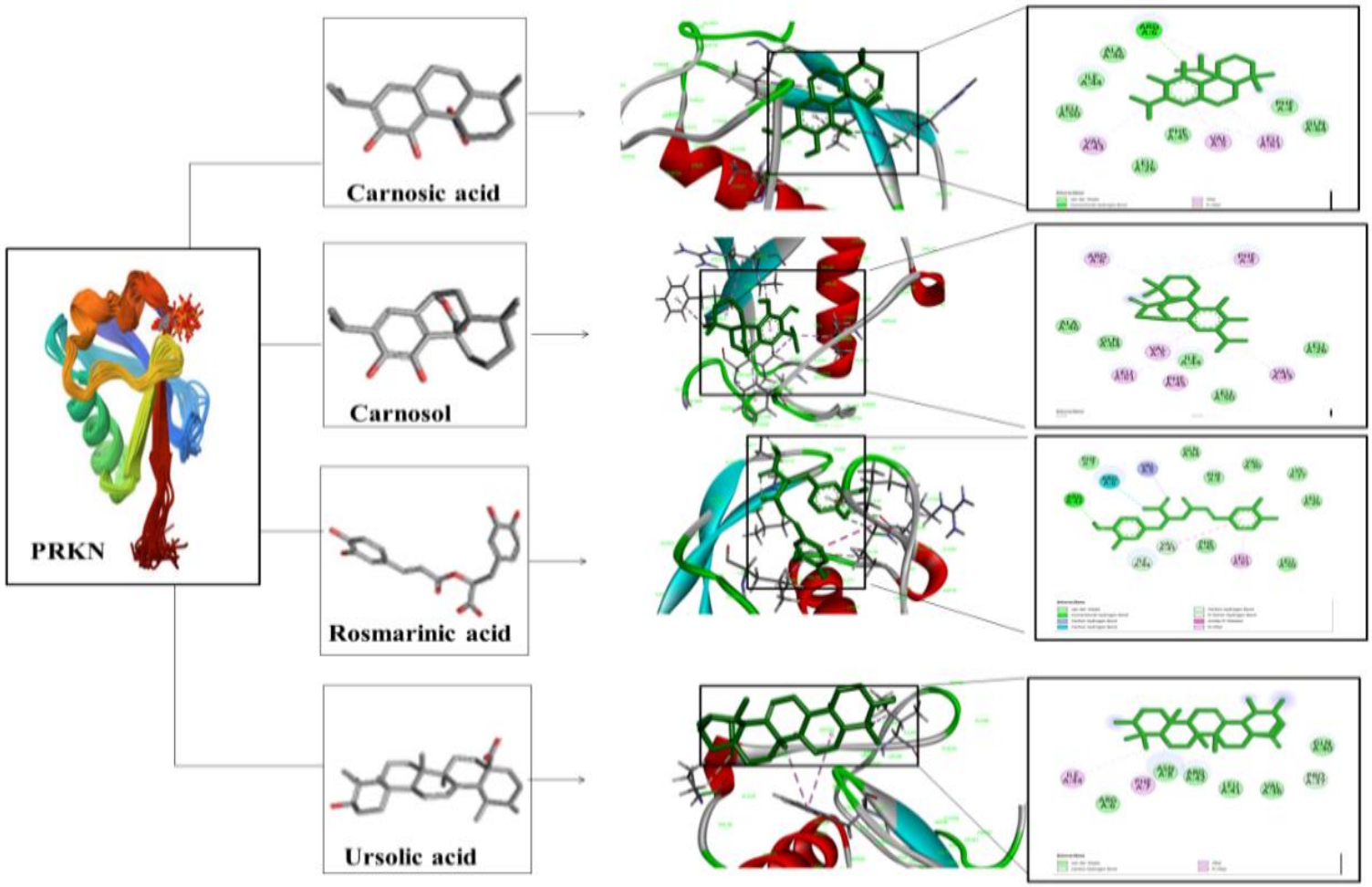


Fig 4.13 A 2D and 3D interactions of PRKN (PDB ID: 5trf) with Bioactive compounds of *R.officinalis*

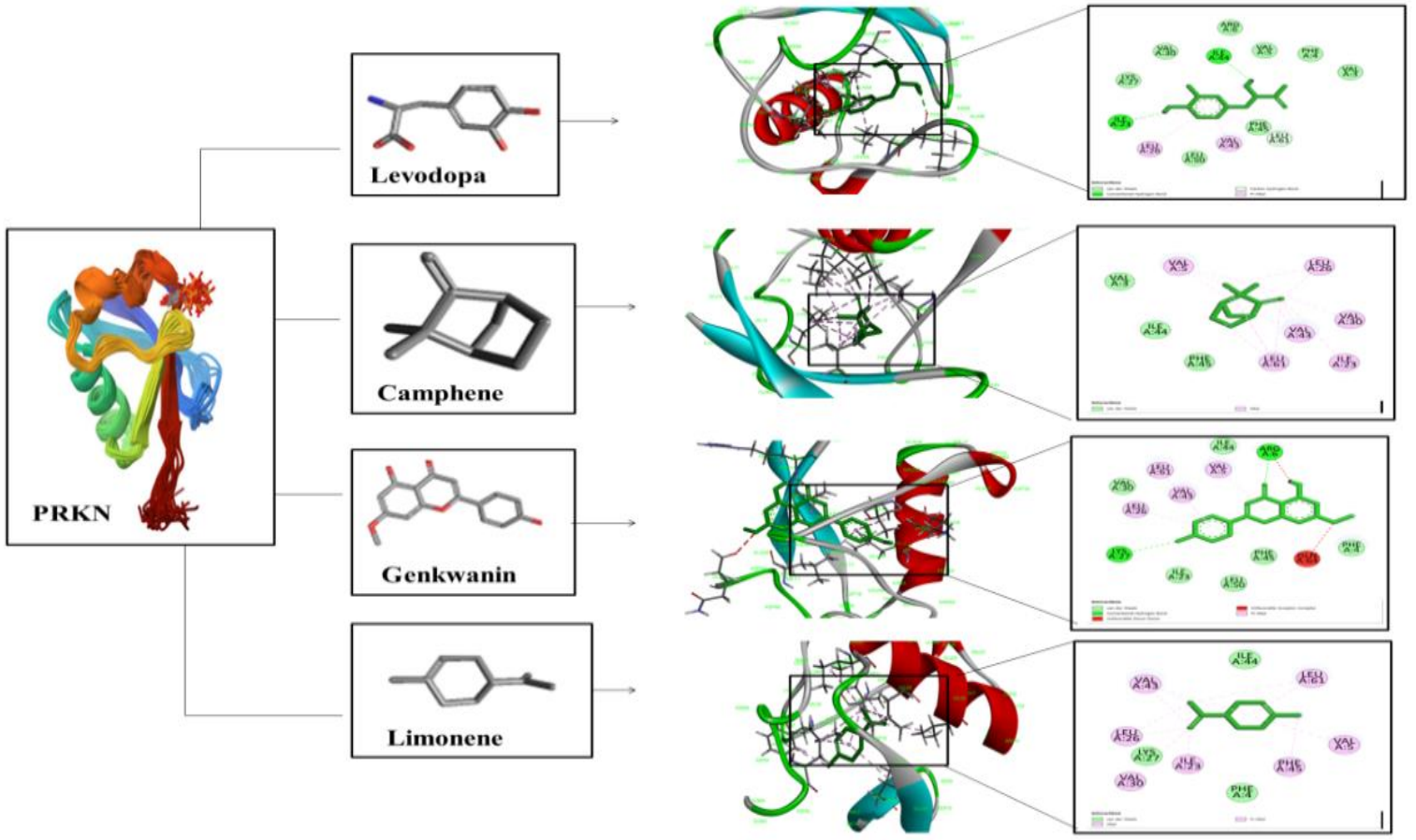


Fig 4.13 B 2D and 3D interactions of PRKN (PDB ID: 5trf) with Bioactive compounds of *R.officinalis*

4.6.1.4 UA exhibits strong binding interactions with IL-6

UA exhibited the highest binding affinity with IL-6, demonstrating a binding energy of -7.9 and forming significant Van der Waals interactions with key residues. CA, with a binding energy of -7.1, established Van der Waals. Similarly, carnosol, with a binding energy of -7.2, showed Van der Waals interactions, while also forming H-bond interactions. In comparison, levodopa, with a binding energy of -5.7, exhibited relatively weaker interactions with IL-6, primarily involving Van der Waals, indicating less stability and fewer favorable interactions than the other ligands.

(Figure 4.14 A-B)

4.6.1.5 RA exhibits strong binding interactions with TNF-α

RA demonstrated the highest binding affinity with TNF-α, with a binding energy of -9.1, characterized by extensive hydrogen bonding, Van der Waals forces, and Pi-anion interactions. Genkwanin, with a binding energy of -8.6, also showed strong binding, primarily through hydrogen bonds and Van der Waals interactions. UA, exhibiting a binding energy of -7.5, whereas Carnosol (-6.5) and CA (-6.3) showed moderate binding affinities, with both ligands displaying Van der Waals and hydrogen bonding. Levodopa, the standard drug, with a binding energy of -6.9, showed moderate binding through hydrogen bonds, Pi-alkyl interactions, and Van der Waals forces, but it was less stable compared to RA, genkwanin, and UA. (Figure 4.15 A-B)

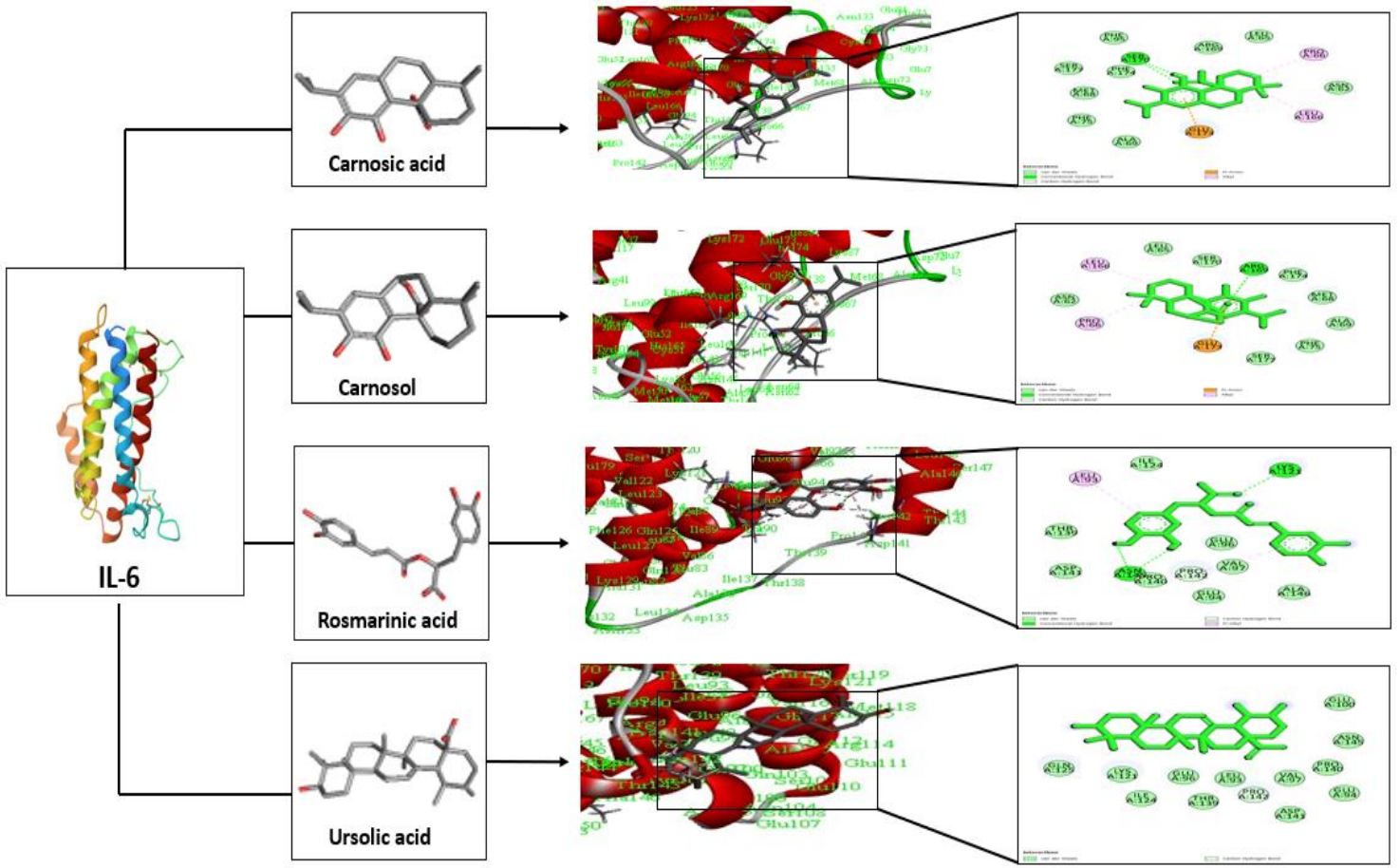


Fig 4.14 A 2D and 3D interactions of IL-6 (PDB ID: 1IL6) with Bioactive compounds of *R.officinalis*

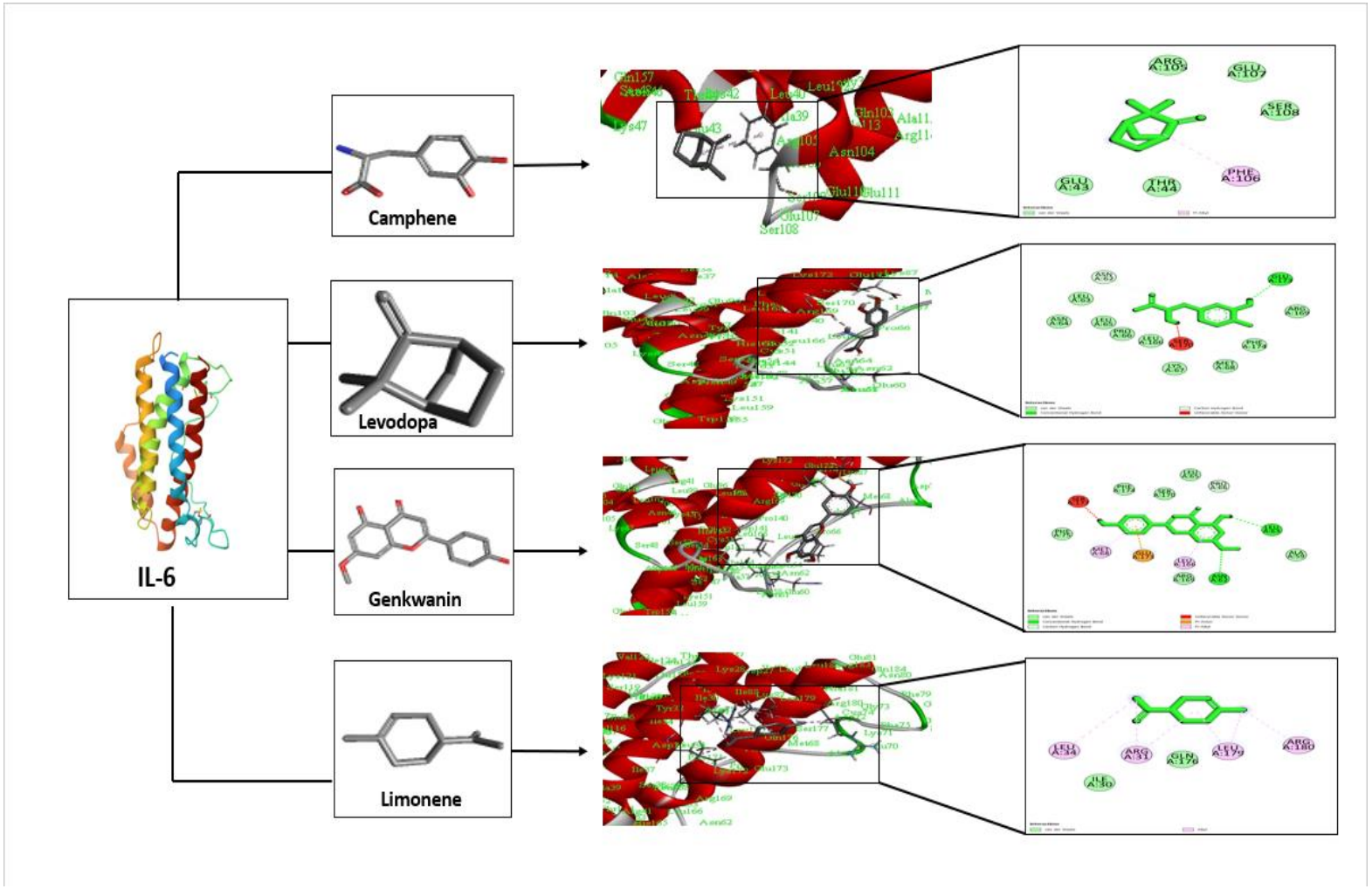


Fig 4.14 B 2D and 3D interactions of IL-6 (PDB ID: 1IL6) with Bioactive compounds of *R.officinalis*

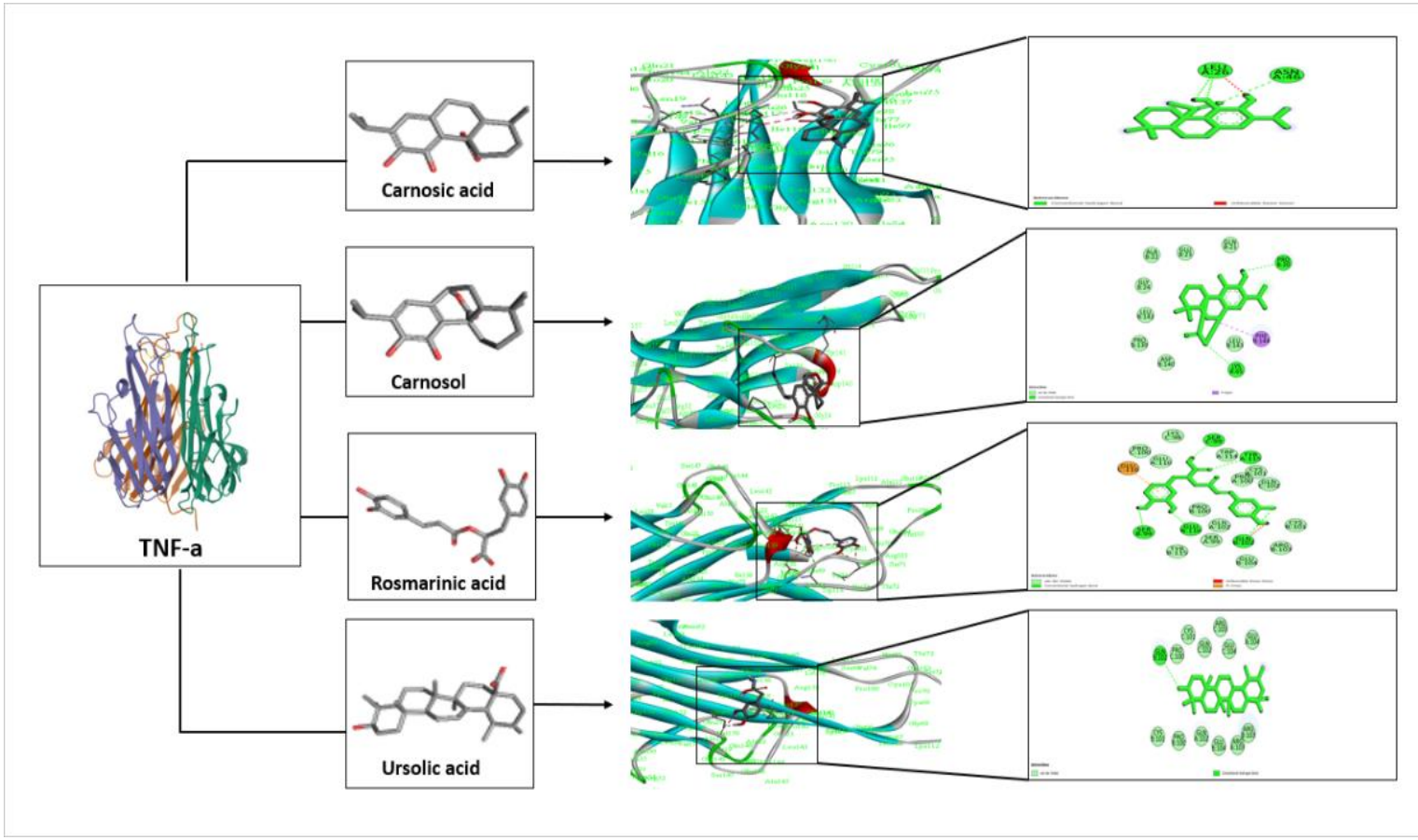


Fig 4.15 A 2D and 3D interactions of TNF-α (PDB ID: 1TNF) with Bioactive compounds of *R.officinalis*

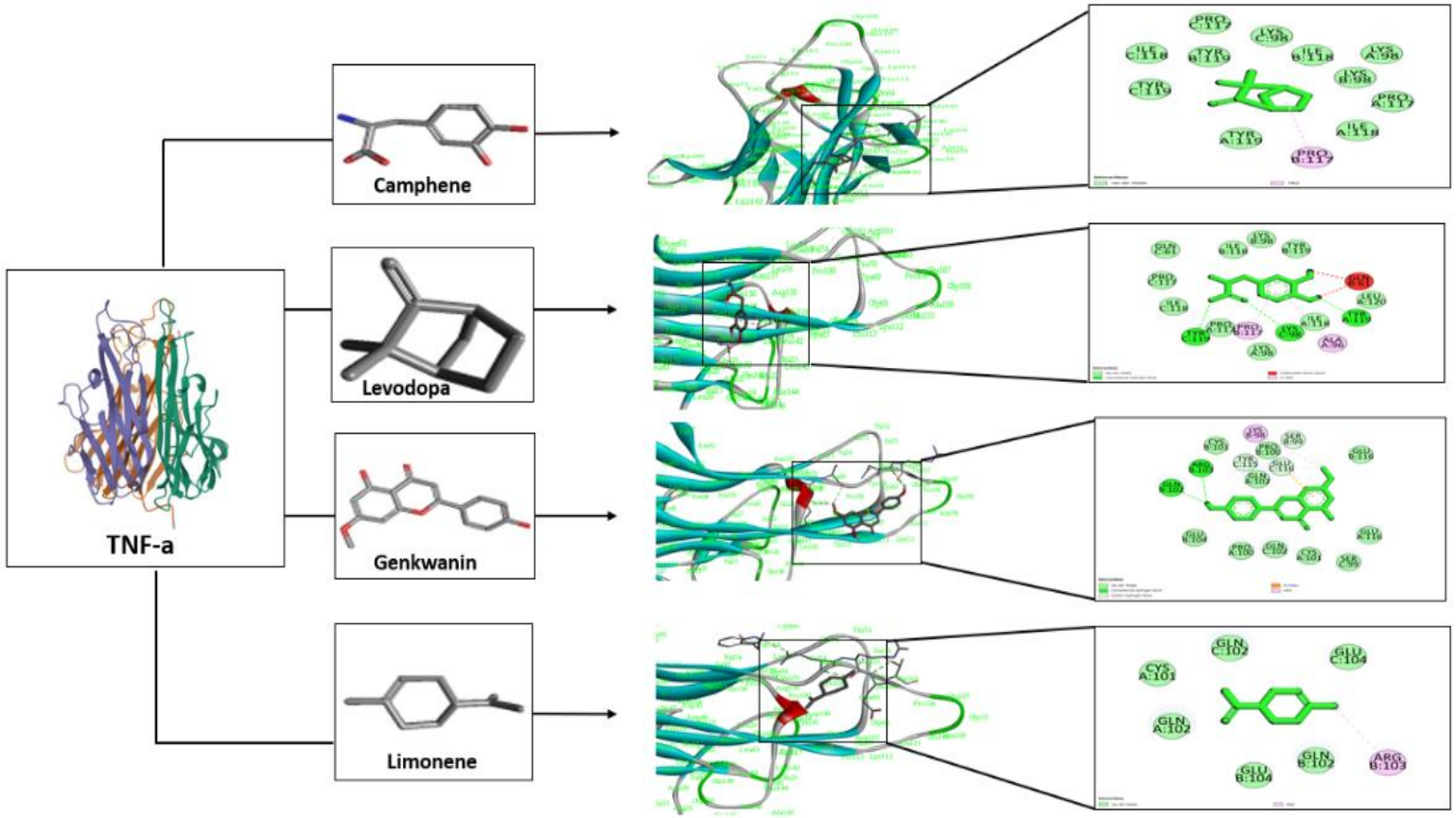


Fig 4.15 B 2D and 3D interactions of TNF-α (PDB ID: 1TNF) with Bioactive compounds of *R.officinalis*

4.6.1.6 UA exhibits strong binding interactions with SOD-1

UA showed the highest binding affinity with SOD1, displaying a binding energy of -6.0 and making various Van der Waals interactions along with C-H bond formation, indicating strong stabilization within the active site. CA and carnosol both had binding energies of -5.3 and -5.8, respectively, with similar Van der Waals interactions and additional alkyl contacts contributing to their moderate binding. In comparison, levodopa exhibited a lower binding energy of -4.5, showing weaker hydrogen bonding and Van der Waals interactions with SOD1, suggesting it has poorer interaction potential compared to the other ligands.(Figure 4.16 A-B).

4.6.1.7 UA exhibits strong binding interactions with SOD-2

UA exhibited the strongest binding affinity with SOD2, with a binding energy of -10.5, forming multiple hydrogen bonds and Van der Waals interactions, indicating a stable binding. CA and carnosol followed with binding energies of -9.0 and -8.7, showing moderate hydrogen bonding, Van der Waals, and C-H interactions. RA and genkwanin also displayed relatively strong binding, while levodopa, with a binding energy of -6.1, formed weaker hydrogen bonds and Van der Waals interactions, indicating a lower binding affinity with SOD2 compared to the other compounds. (Figure 4.17A-B)

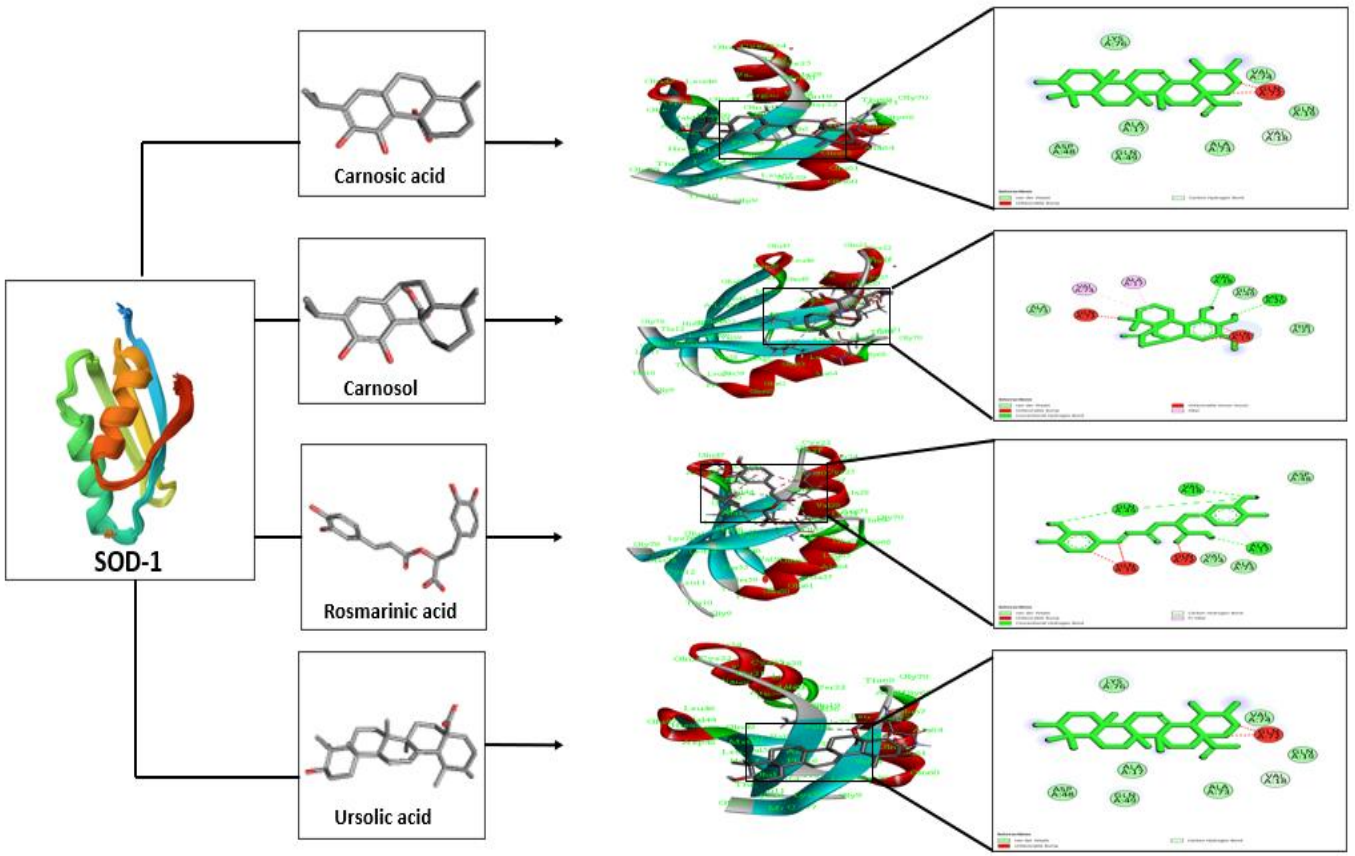


Fig 4.16 A 2D and 3D interactions of SOD-1(PDB ID: 2RSQ) with Bioactive compounds of *R.officinalis*

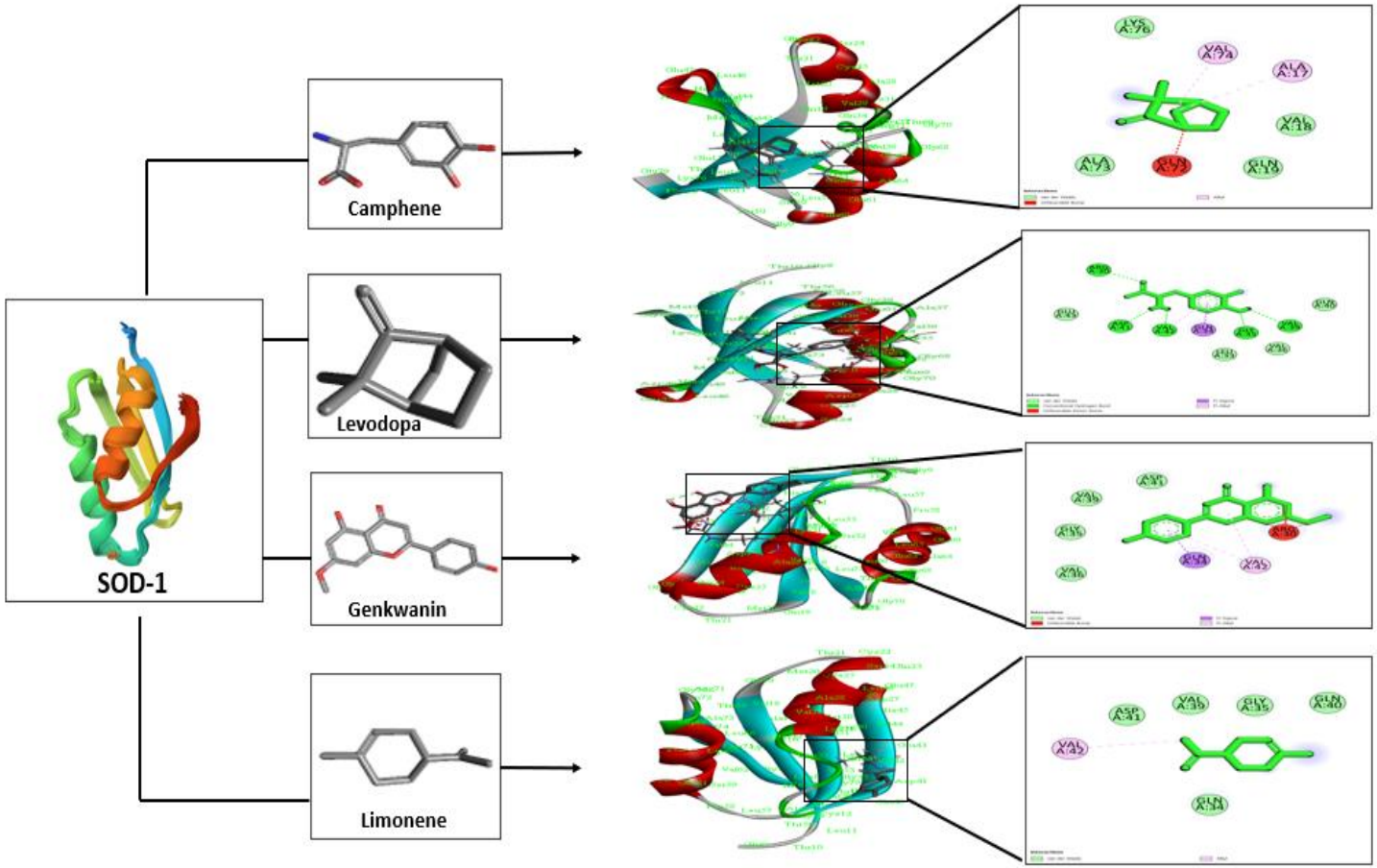


Fig 4.16 B 2D and 3D interactions of SOD-1(PDB ID: 2RSQ) with Bioactive compounds of *R.officinalis*

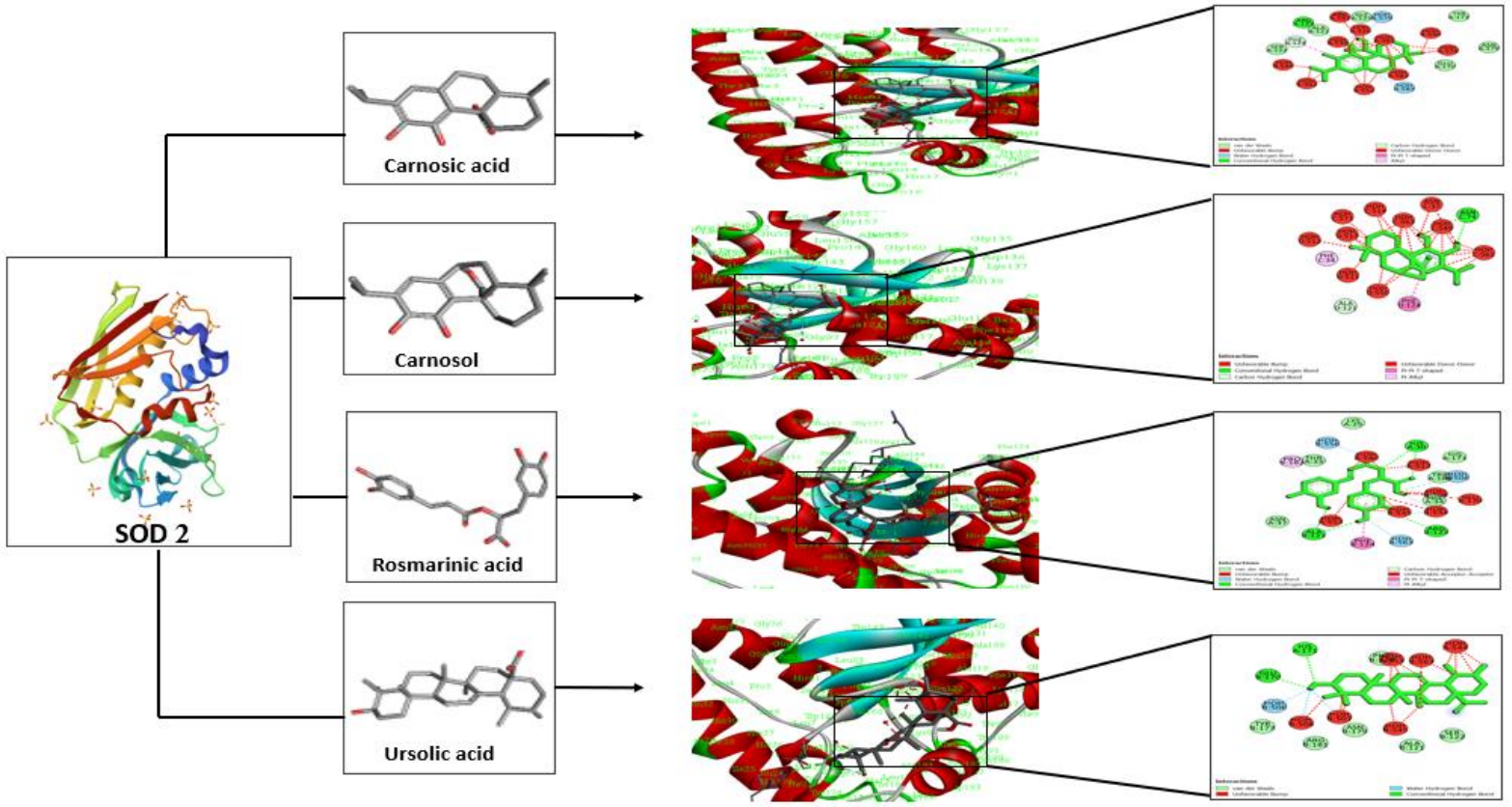


Fig 4.17 A 2D and 3D interactions of SOD-2 (PDB ID:1EN5) with Bioactive compounds of *R.officinalis*

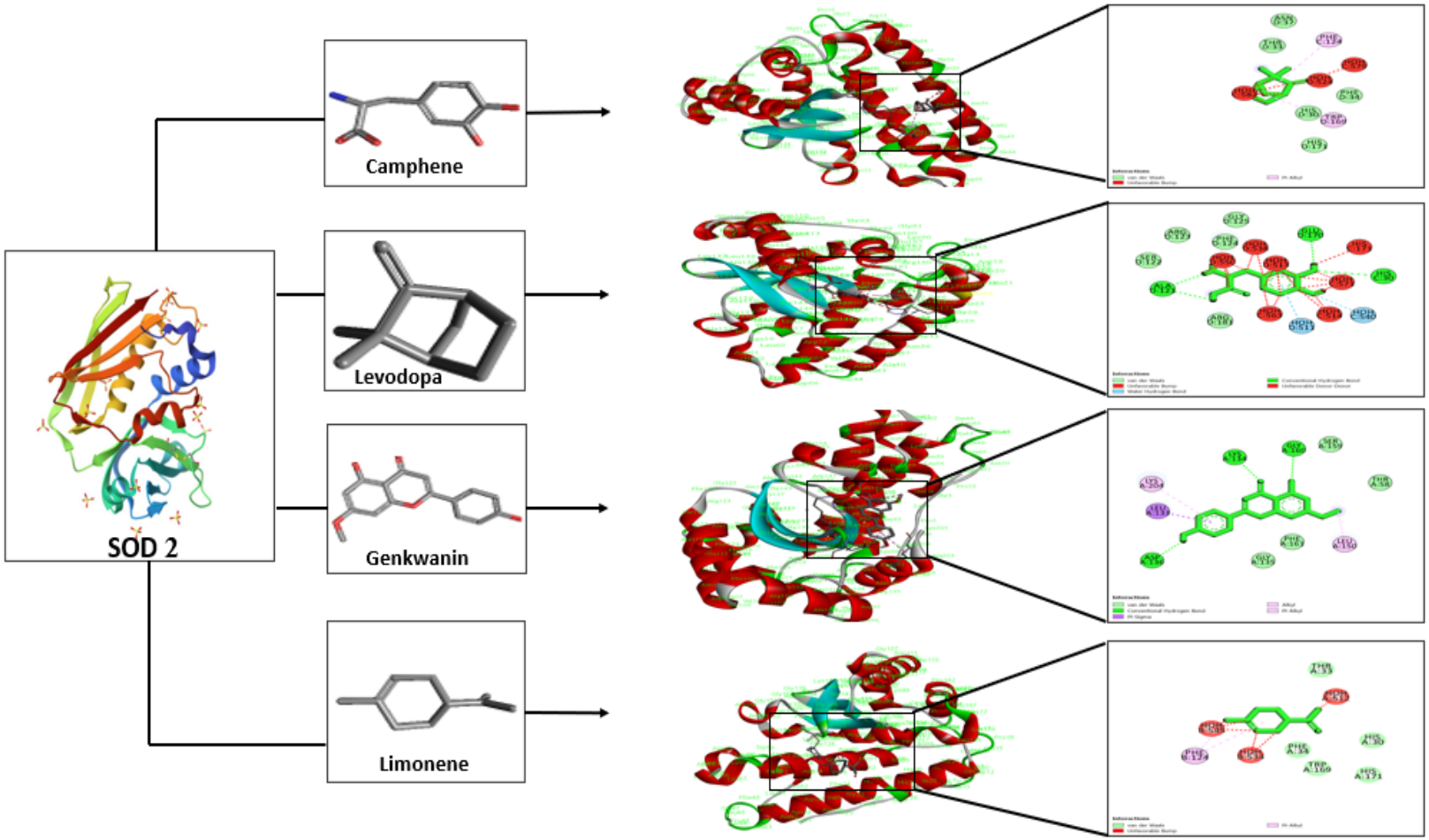


Fig 4.17 B 2D and 3D interactions of SOD-2 (PDB ID:1EN5) with Bioactive compounds of *R.officinalis*

4.6.2 Drug likeness analysis of all the bioactive compounds of *R. officinalis*

The drug likeness of the compounds was determined using a Lipinski filter, ADMETSAR, and SwissADME. These tools were used to analyze the compounds' drug-like qualities and evaluate their pharmacokinetic properties. All compounds demonstrated features that suggest they have the potential to be utilized as medicinal treatments. Their molecular weights were below 500 Daltons, and they exhibited fewer than five hydrogen bond donors and fewer than 10 acceptors. The molar refractivity and cLogP results were consistent with the Lipinski rule. The compounds exhibited similar outcomes in all other aspects of the Lipinski filter, ADMETSAR, and SwissADME, indicating their drug-like properties and their usefulness as treatment agents for AD in Table 4.2.

Most of the compounds comply with the key Lipinski rules, except for UA, which exceeds the recommended molecular mass and LogP values. Camphene and Limonene exhibit low hydrogen bond donor and acceptor counts, which might make them less interactive, but also more permeable. These results suggest that most compounds, especially Carnosol, Genkwanin, CA and RA, could be considered for further drug development due to favorable drug-likeness properties. Table 4.3 compares various biological and toxicological properties of these compounds.

Table 4.2 Lipinski Filter analysis

Lipinski filter	CA	RA	UA	Carnosol	Genkwanin	Camphene	Limonene
Molecular mass g/mol	332.43	360.31	456.70	330.42	284.26	136.23	136.23
H bond donors	3	5	2	2	2	0	0
H bond acceptors	4	8	3	4	5	0	0
Log P (cLOGP)	2.93	1.17	3.71	2.97	2.48	2.58	2.72
Molar Refractivity	95.43	91.40	136.91	92.83	78.46	45.22	47.12

Table 4.3 ADMET properties

Parameters	CA	RA	UA	Carnosol	Genkwanin	Camphene	Limonene
BBB	+	+	+	_	_	+	+
HIA	+	+	+	+	+	+	+
CaCo-2 permeability	+	_	+	+	_	+	+
AMES toxicity	_	_	_	_	+	_	_
Carcinogens	_	_	_	_	_	_	_
AOT	|||	|||	|||	|||	|||	|||	|||
SCL	Mitochondria	Mitochondria	Mitochondria	Mitochondria	Mitochondria	Lysosome	Lysosome

(BBB= Blood Brain Barrier; HIA= human Intestinal Absorption; AOT=Acute Oral Toxicity; SCL= Sub cellular Localization; + presence; - absence)

Discussion

In this study, we establish PD mouse model with MPTP at a dose 30 mg/kg (i.p.) for five consecutive days. The MPTP model, which has been widely used to replicate the dopaminergic neuron loss in PD, also bring oxidative stress, neuroinflammation, and motor dysfunction corresponding to human disease (Jackson-Lewis & Przedborski, 2007). In choosing this approach, our model is in corelation with other models in the study of PD that has employed MPTP with similar neurodegenerative impact on the substantia nigra pars compacta (Blesa & Przedborski, 2014) and reduce dopamine levels in the striatum.

The therapeutic intervention was started after 5 days from MPTP exposure so that the PD-like condition can be developed. *R. officinalis* extract was given intraperitoneally at a dose of 120 mg/kg and *Bacillus clausii*, at 2x10^4 CFU for seven consecutive days. This dosage as well as the treatment duration chosen are consistent with earlier studies where *R. officinalis* and probiotics were shown to prevent neurodegeneration through antioxidation as well as anti-inflammatory properties (Paparo et al., 2020). This timeline was selected as prior studies have indicated that shorter and more selective protocols have the desired impact and both *R. officinalis* has been shown to modulate antioxidative signals, whereas *Bacillus clausii* helps to maintain the relationship between the gastrointestinal tract and the brain by decreasing inflammation (Mulak & Bonaz, 2015).

The use of *R. officinalis* and *Bacillus clausii* in the present work was done for the first time with our PD mice model while previous findings worked with these compounds separately. We hypothesized that when these interventions are combined, they will synergistically enhance neuroprotection based on their antioxidative, anti-inflammatory and microbiome restoring effects. This is in accordance with recent studies in the

treatment of PD that has noted the use of multi-modal therapeutics to combat the multiple pathways of neurodegenerative diseases (Hirsch & Hunot, 2009).

Our protocol, centring on the central (neural) and peripheral (gut microbiome) interventions, captures future therapeutic models of the neuro-immune and gut-brain axis. Such integrated models have implications for protracting treatment effectiveness as they are potentially able to address neuroinflammation and gut microbiota alteration simultaneously,whose directions are gradually gaining popularity within neurodegenerative disorders (Sampson et al., 2016).

In current study, the enhanced locomotor and exploratory movements were observed in MPTP induced mice after the administration of *R. officinalis* and *B. clausii* individually and in combination treatment groups.

The open field test measured the exploratory behavior of mice by recording movement in central and peripheral areas. MPTP treatment alone resulted in significantly reduced activity in both areas, indicating the expected reduced locomotor activity. Treatment with *R. officinalis* and *B. clausii* reversed these deficits, with the combined treatment group showing the highest recovery in central and peripheral zone crossings. This outcome suggests a strong locomotor and motor-enhancing effect, consistent with studies that show *R. officinalis* can enhance locomotor behaviors through its anti-inflammatory effects and that *B. clausii* may support motor function by stabilizing the gut-brain axis and reducing systemic inflammation (Tursi et al., 2013).

These findings align with other studies using natural compounds to combat PD-induced anxiety and motor deficits. For instance, recent research on herbal compounds with antioxidative properties, such as curcumin, has shown similar improvements in exploratory behavior in PD models (Rezaei Kamelabad et al., 2021). However, our study extends these findings by demonstrating that the combination of *R. officinalis* and

B. clausii produces a more pronounced effect, supporting the hypothesis that combined therapeutic approaches may offer enhanced efficacy in PD models.

The Pole Test, which assesses bradykinesia and motor coordination, revealed significant motor impairments in MPTP-treated group, indicated by longer descent times. Both *R. officinalis* and *B. clausii* treatments reduced descent times, with the combination treatment group showing the most substantial improvement. Earlier research has shown that compounds that possess antioxidant and anti-inflammatory effects can alleviate bradykinesia in PD animal models (Sedelis et al., 2001). These studies parallel our results; thus, this study cconfirms that both *R. officinalis* and *Bacillus clausii* alleviate motor dysfunction due to the suppression of oxidative stress and neuroinflammation, two critical aspects in PD development .

Other comparative studies, including research with natural remedies for example ginseng, also show that bradykinesia increases (Ratan et al., 2021), Yet it often misses critical element of gut-brain axis modulation and thus represents a classic example of *B. clausii* probiotic. This not only promote the motor function recovery, but also normalise the systemic inflammation through microbiome intervention, hence the potential for dual-function remedy for PD symptoms.

The cylinder test measured the lateral bias in forelimb usage, which is normally affected by degeneration of dopaminergic neuron in PD. While the MPTP-treated group exhibited a marked asymmetry, there was a significant decrease in this rotational asymmetry in animals treated with *R. officinalis* and *B. clausii.* These observations correlate with neuroprotective properties of *R.officinalis* that exerted successful actions against dopaminergic lesion in other experimental models of PD (Magno et al., 2019). *B.clausii*, on the other hand, improves motor function by preserving the homeostasis of

the neuroinflammation that has been noticed in recent experiments on probiotics in neurodegenerative models (Mulak & Bonaz, 2015).

In general, the behavioral studies presented in this study suggest that the possible combined treatment with *R. officinalis* and *B.clausii* not only prevents motor dysfunction in PD patients but also contributes to increased performance in the exploratory activity. This makes the present multi-compound therapy more effective than single-compound therapy and may be applied to future integrative therapies for PD. The role of these combined treatments observered in this study should be qualitatively assessed in future research and behaviors tests should be added to prove these findings in other models.

Histopathological assessment in the present study was carried out to understand the neuroprotective effects of *R. officinalis* and *B. clausii* on the brain tissues in the MPTP-induced PD mouse model. After treatment, the present study evidenced substantial neuron-saving consequences and decline in neuronal degeneration mainly in the combined treatment group. These changes were evaluated by H&Estaining to determine dopaminergic neuronal count in substantia nigra and hippocampal regions that are normally affected by neurodegeneration in PD.

In the MPTP-treated group, decreased density and a significant loss of neurons was observed in the hippocampus and SNpc, as it is typical of the prior PD models where MPTP causes degeneration of dopaminergic neurons (Jackson-Lewis & Przedborski, 2007). Treatment with *R. officinalis* alone showed noticeable protection of dopaminergic neurons, reducing cellular damage through its antioxidative and anti-inflammatory properties. Studies on *R. officinalis* have demonstrated similar neuroprotective effects due to bioactive compounds like RA and CA, which counteract oxidative stress and inhibit pro-inflammatory cytokine release. These findings suggest

that *R. officinalis* can mitigate dopaminergic neuronal death, likely by neutralizing ROS and downregulating inflammatory pathways.

B.clausii, while less directly related to central nervous system protection, demonstrated beneficial effects by maintaining neuronal structure through the gut-brain axis. As noted in recent studies, *B. clausii's* probiotic role includes stabilizing systemic inflammation, which in turn contributes to neuroprotection in degenerative models (Mulak & Bonaz, 2015). The combination treatment exhibited the highest preservation of neuronal integrity, supporting the hypothesis that combined antioxidant and anti-inflammatory effects through central and peripheral mechanisms offer greater neuroprotection.

Gene expression analysis in this study provided insights into the molecular mechanisms by which *R. officinalis* and *B. clausii* confer neuroprotective effects in the MPTP-induced PD model. A higher level of IL-6 and TNF-α in the MPTP-diseaded group established the neuro inflammation in PD helping in neuronal damage. Our results were in line with previous investigations, which revealed that *R. officinalis* has potent anti-inflammatory effects accompanied by the ability to inhibit microglial activation and decrease cytokine levels including IL-6 and TNF-α . Likewise, *B. clausii* lowered IL-6 and TNF-α productions likely through alteration of gut microbiota, which decrease systemic inflammation – a mechanism recently proposed as a therapy for neurodegenerative diseases.

The IL-6 and TNF-α levels were again significantly lowered in the combination therapy group indicating that the synergism between *R. officinalis* and *B. clausii* offers better anti-neuroinflammatory benefits than independent use of the therapeutic agents. This outcome is in concordance with research on multiple anti-inflammatory approaches that address multiplicity of the inflammation sources; this is a better approach towards

treatment of PD (Tursi et al., 2013). Since neuro-inflammation is a major feature in PD and is critical to the progression of the disease, the current study's findings indicate that such combined treatments could slow down the progression of PD through mitigating inflammation on dopaminergic neurons.

The present study reveals that MPTP treatment decreased the SOD1 and SOD2, which may cause the cells to become more sensitive to oxidative stress, which correlates with the existing studies that due to the high metabolic rate, dopaminergic neurons in the substantia nigra are especially sensitive to ROS. The stimulation of antioxidant enzymes SOD1 and SOD2 was observed, and it may be mediated by bioactive compounds present in *R. officinalis*, such as CA, which activated the Nrf2 pathway (de Oliveira et al., 2019). *B. clausii* treated group increased SOD levels only slightly, implying that its action on oxidative stress might be due to its immunomodulatory effects on systemic inflammation as shown in gut-brain axis-related investigations (Paparo et al., 2020).

Thus, the combinational treated group of *R. officinalis* and *B.clausii* showed a synergistic effect on the SOD1 and SOD2 genes expression level, upregulated higher than both treatments separately. The present result is consistent with accumulating evidence that suggests that multi-compound therapies may modulate oxidative stress through multiple protective signaling mechanisms better (Prendecki et al., 2020c).

The results from this study reveal that *R. officinalis* and *B. clausii* have potential neuroprotective effects because genes related to oxidative stresses and inflammation are suppressed in this study. These treatments increase the overall antioxidant gene expression and at the same time decrease the expression of pro-inflammatory cytokines, both of which are important causes of neurodegeneration in PD. This approach is

comparatively beneficial in designing integrative therapies that take advantage of both central and peripheral routes in countering neurodegeneration.

The in-silico analysis in this study aimed to explore the molecular interactions and binding affinities of key bioactive compounds in *R. officinalis* (such as CA, UA, RA, carnosol, genkwanin, limonene, and camphene with PD-related proteins, specifically SNCA, PRKN, APOE, IL-6, TNF-α, SOD1, and SOD2.

Docking results indicated that several compounds exhibited strong affinities for PD-related targets. UA displayed the highest binding affinities with SNCA, PRKN, and APOE, followed by CA and carnosol, suggesting potential for these compounds to stabilize PD-related proteins against oxidative stress and neuroinflammation. SNCA implicated in synaptic dysfunction and aggregation demonstrated remarkable binding with UA and carnosol at -7.0 kcal of binding energy. These interactions indicated these compounds as potential bioactive compounds to affect SNCA stability and aggregation. The basic mechanisms in PD pathogenic protein in neurodegeneration, had the highest binding energy for UA at -7.1 kcal/mol, with CA and carnosol at -6.8 kcal/mol. This strong interaction suggest that these compounds might attenuate APOE-mediated neuroinflammation and it might exacerbate the accumulation of alpha-synuclein, which is implicated in PD, although this hypothesis remains under study(Gao et al., 2011). We found favorable results with the PD protein PRKN, which is crucial for mitochondrial dysfunction; our findings imply that UA and genkwanin could help maintain mitochondria by boosting PRKN activity in PD (Valente et al., 2004)

As demonstrated in the drug-likeness assessment of the bioactive compounds derived from *R. officinalis*, most of the extracted compounds confirm to Lipinski Filter and ADMET properties for the use in therapeutic products. Similarly, previous research on similar compounds from other medicinal plants serve to explain these findings. For

example, the complexes such as carnosol and genkwanin, based on the Log P and hydrogen bonding possibilities, still keep balance and are close to the successful drug substances, which were investigated in the literature earlier to be characterized by particular Log P and hydrogen bonding for their successful absorption and bioavailability (Kock & Brown, 2020).

Additionally, the compounds under discussion possess high BBB permeability and high HIA, which can be considered advantageous for further practical application. This is in concordance with prior studies emphasizing that BBB permeability for molecules for CNS disorder treatment is indeed critical (Davis et al., 2019). The non toxicity to most of the AMES compounds especially genkwanin also supports the safe therapeutic compounds that where also supported in other studies that showed that lower toxicity yields higher rates of clinical success.

The subcellular localization of these compounds, primarily targeting mitochondria, suggests potential mechanisms of action that could be exploited in drug development, similar to the mitochondrial targeting employed by certain anti-cancer and anti-inflammatory drugs currently in the market. This aspect of drug targeting is crucial as it supports the compounds' roles in modulating key metabolic and signaling pathways within the cell, potentially leading to innovative treatments for a range of diseases (Chatterjee et al., 2022). It can be suggested that the drug-likeness of the compounds from *R. officinalis* closely matches the features of the oral drugs.

Conclusion

This study shows that *R. officinalis* and *B. clausii* have potential therapeutic effects in PD through antioxidative and anti-inflammatory actions in MPTP-induced PD mouse models. Hence, the synergistic effects of *R. officinalis* and *B. clausii* led to amelioration of motor deficits and enhanced locomotor activity reinforcement of dopaminergic neurons, and decreased neuroinflammation and increased antioxidant gene expressions better than mono-therapeutic approaches. The molecular docking analysis additionally underscored the strong binding profile for bioactive compounds in *R. officinalis* with the PD-related proteins to explain the neuroprotective impact. These findings correlate with recent outlooks on combined and multiple active compound approaches towards neurodegenerative disorders, where treating both neuropathological and systemic, and inflammatory components is beneficial. This synergistic approach can be further developed in future research by including other bioactive agents, by investigating the effects on different doses of the substances and by proving the effects of the substances on PD patient sample data. Additionally, studies conducting clinical trials of *R. officinalis* and *B. clausii* alongside putative treatments for PD may assist in the determination of usage, feasibility, and overall complementary approaches to PD and other neurodegenerative diseases treatment.

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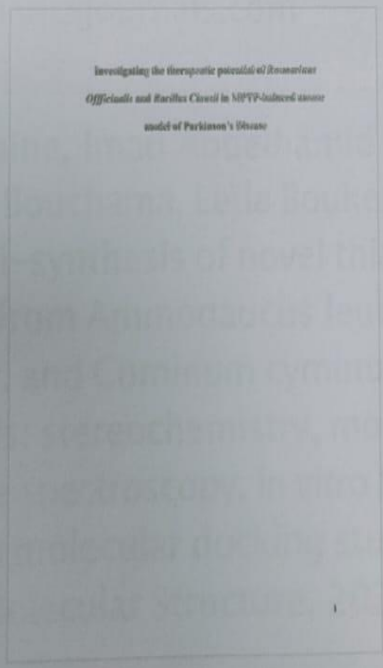

Investigating the therapeutic potential of *Rosmarinus Officinalis* and *Eucalbus Citrosi* in MPTP-induced mouse model of Parkinson's Disease

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