1. Introduction

The principal characteristic of Parkinson's disease (PD) is a decline of dopaminergic neurons in the substantia nigra's pars compacta. This progressive neurodegenerative condition causes various motor-related issues such as resting tremors, bradykinesia, stiffness, and postural instability. Exhibiting a frequency of 1% to 2% among those over 60 years of age, PD is possibly the neurological condition with the greatest rate of growth in the globe (1).

The main goal of PD therapy now available is symptomatic alleviation. Levodopa, a substance known as dopamine precursor that helps regain the availability of dopamine within the brain, is the foundation of PD treatment. MAO-B inhibitors, COMT inhibitors, and dopamine agonists are a few other pharmacological therapies (2, 3). To treat symptoms, alternatives to medicine including physical therapy and deep brain stimulation (DBS) are frequently used (4). But there are a lot of drawbacks to these therapies. Despite its effectiveness, levodopa frequently causes dyskinesias and changes in its effectiveness gradually. Dopamine agonists may result in adverse effects such as insomnia, hallucinations, and problems with impulse control. Furthermore, the primary focus of these therapies is on motor symptoms; the illness advancement is not stopped. Therapies that can alter the course of the illness and offer complete symptom relief are desperately needed (5, 6). Due to the combined influences of environmental and genetic variables (e.g., SNCA, LRRK2, and Parkin), the majority of PD patients most likely have a multifactorial origin. While some lifestyle factors may lessen the risk, exposure to toxicant substances (like pesticides) and head injuries may raise the chance of Parkinson's disease (PD). Environmental exposures might have different impacts depending on genetic susceptibility characteristics (7).

Of these, more recent studies have brought attention to the crucial role the gut-brain axis (GBA) plays at the beginning of the course of PD (8, 9, 10, 11). The brain and the gastrointestinal system

(GI) communicate in both directions through neuronal, endocrine, and immunological pathways, which is called the GBA. The intricate collection of bacteria called the gut microbiota, that lives in the GI tract, is essential to preserving homeostasis in this axis. A dysbiosis, or an unbalance in the microbiota of the gut, relates to a diverse enumerate of mental and neurological conditions, including PD (12, 13). Research has demonstrated that, in comparison to healthy controls, individuals with PD frequently have notable alterations in the makeup of their gut microbiota, raising the possibility of a connection between gut health and neurodegeneration (14, 15).

Leaky gut syndrome is one way that gut dysbiosis may aggravate Parkinson's disease. Bacterial endotoxins, like lipopolysaccharides (LPS), may get into the bloodstream, resulting in raised intestinal permeability. This sets off a chain reaction of systemic inflammation, which may have a detrimental effect on the CNS and perhaps exacerbate neurodegenerative disorders (16, 17). Increased-than-normal levels of pro-inflammatory cytokines). (i.e., IL1β, IL6, TNFα) have demonstrated that systemic inflammation plays a major part in the development of PD (18).

Experimental models have yielded important insights into the GBA in PD. For example, the pesticide rotenone is administered in the rotenone-induced PD models, that has been extensively used to investigate the illness. Exposure to rotenone mimics the clinical hallmarks of PD by causing mitochondrial malfunction and oxidative stress. Interestingly, gut dysbiosis and increased intestinal permeability are also shown in rotenone-induced PD mice, supporting the idea indicates the pathogenesis of the disease involves the gut in a major way (19, 20, 21).

These results have been further supported by research on humans, which shows that gastrointestinal symptoms, including constipation, are frequently experienced by PD patients well prior to the onset of motor signs. This time correlation raises the possibility that gastrointestinal alterations might act as early indicators for Parkinson's disease (PD), providing new opportunities

for early detection and treatment (22, 23). In addition, there is a tremendous deal of interest in the potential for targeting the GBA for treatments. The effectiveness of several approaches, including fecal microbiota transplantation (FMT), probiotics, prebiotics, and dietary adjustments, in modifying the gut microbiota and reducing PD symptoms is being investigated (24, 25, 26).

Mucuna pruriens (Mp) is a member of the Fabaceae family and is both a yearly and perennials legume with a variety of medicinal uses. Among Mp's principal actions are those that are anti-oxidative, anti-inflammatory, anti-epileptic, anti-microbial, etc. It's used extensively as a strong aphrodisiac. Since the 19th century, researchers have studied Mp's anti-Parkinsonian action. Numerous investigations have demonstrated Mp's neuroprotective properties. The key component in charge of Mp's anti-Parkinsonian action is levodopa, or L-DOPA. In addition to L-DOPA, a number of other significant bioactive substances, such as betulinic acid (BA) and ursolic acid (UA), oligosaccharide and phenolic compound also show comparable neuroprotective properties (27, 28).

The goal of this research is to look at how gut dysbiosis, increased intestinal penetrability, and systemic inflammation impart to PD and to evaluate potential treatments targeting the GBA using in-silico approaches such as network pharmacology, *in-vitro,* and *in-vivo* models. Specifically, we will look at *Mucuna pruriens* dual advantages for prebiotic activity and levodopa content, which might provide a unique method to manage PD by modulating gut health.

Literature review

This section emphasizes the current research trends and how this research can be useful in making the hypothesis. Recent studies have demonstrated the significance of the GBA and the colon's participation in the pathophysiology of PD, especially in models when the pesticide rotenone is

used. This study addresses possible gut-targeting therapy options and highlights the latest findings on the pathways connecting the colon to PD (29). The GBA and the colon's influence on the development of PD have been highlighted in recent research, particularly in models when the pesticide rotenone is used (30). This section addresses possible gut-focused treatments and outlines new findings about the connections between PD and the colon.

2.1. Microbiota-gut-brain axis and gut dysbiosis

The reciprocal contact between the CNS and the GIT is described as the "gut-brain axis (GBA)." The crucial role of the GBA in the context of PD is becoming increasingly widely recognized. In line with recent studies, such interactions are greatly impacted by the microbiota in the stomach. The link between microbiota and GBA seems to be bidirectional and involves communication via neurological, endocrine, immunological, and humoral connections between the brain and the gut microbiome, as well as between the brain and the gut microbiome (GM) (31). A common feature of people with PD is dysbiosis of the gut microbiota, which may have a role with regard to motor and nonmotor signs and symptoms (21). The gut microbiota's composition and functions are continually changing, which are highly influenced by genetic and environmental variables (infection, medicine, diet, etc.), and aberrant quantities or quality are referred to as intestinal microbiota disorders (12). Lipopolysaccharides (LPS) were increased and short-chain fatty acids (SCFAs) were lowered, indicating a disturbance of the gut microbiome in PD patients, increasing their susceptibility to pro-inflammatory and neurotoxic bacteria. These changes also affect the gut flora as a whole (32). Furthermore, the gut microbiome can generate microbial amyloid protein, or functional amyloid protein, which may speed up α-synuclein aggregation in the nerve plexus of the gut and transmitted via transsynaptic cell-to-cell transmission toward the CNS. Additionally, it may increase intestinal and systemic inflammation (24). The aforementioned processes may alter

the process that causes neuronal impairment or heighten vulnerability to neuronal damage, leading to the development and growth of PD (33).

2.2. Parkinson's disease-related changes to the predominant gut microbiota and their relationship to clinical features

The clinical attributes of PD, such as disease progression, severity, and clinical symptoms, are intimately connected with the makeup and implication of gut microbiota. The GM alteration in PD individuals continued to be observed in subsequent samples two years later, according to high-throughput sequencing studies. The most noticeable alterations included an increase in the bacterial group that produces LPS., which has pro-inflammatory characteristics, and a reduction in the bacterial group that produces SCFAs, which has anti-inflammatory effects (34, 35, 36, 37). As PD progressed, the prevalence of *Faecalibacterium, Lachnospiraceae, Roseburia, Prevotella*, family, and its important component *Butyrivibrio* declined dramatically. In contrast, the prevalence of *Megasphaera, Akkermansia, Verrucomicrobia,* and *Lactobacillaceae* persisted in multiplying in PD patients. (38). Of it, *Roseburia* broke down carbs to create SCFAs, that may shield the intestines against infections. *Roseburia* depletion is linked to a decline in cognitive function and impacts the host's capacity to heal epithelium and control inflammation. Proteins and carbohydrates are broken down by *Prevotella* to create SCFAs, whose quantity is inversely connected with the severity of the condition. Its abundance is linked to the decline in cognitive function and is markedly reduced in individuals with PD who are developing quickly (39). The drop in butyric acid abundance is associated with motor difficulties and impaired motor function. The excessive accumulation of *Akkermansia* causes harm to the intestinal mucosal barrier and inflammation, which therefore results in aberrant α-synuclein accumulation in the gut. Ultimately, this results in increased endotoxemia and systemic inflammation, which further accelerates the

development of neuropathology (40, 41). Simultaneously, Alterations in the constitution of the gut microbiota might impact neurodegeneration by inducing a particular inflammation reaction; Bacteroides abundance is connected with both the degree of motor distress and plasma TNF-α levels. Furthermore, the decreased abundance of the main butyrate makers (such as the genera Roseburia, Prevotella, and Romboutsia) was connected to the intensity of depression-related symptoms in PD individuals as well as deteriorating neurocognitive performance (42, 43).

2.3. Inflammation and Gut Permeability

Inflammation and increased gut permeability are two major ways that the colon contributes to the mechanisms behind PD. According to studies, intestinal barrier integrity may be weakened by gut dysbiosis, which makes it possible for bacterial endotoxins such as lipopolysaccharides (LPS) to reach the circulation (44). According to human research, PD patients had greater levels of intestinal permeability and LPS-binding protein, which are indicators of systemic endotoxemia. Research indicates that FMT therapy can restore dysbiosis as well as improve the rotenone-induced PD animal model. A major contributing factor in this treatment may be the inhibition of aggravation facilitated by the LPS-TLR4 signaling in both the GI tract and the brain. Furthermore, via the GBA, they demonstrate how rotenone-induced microbiome dysbiosis assists in the onset of PD (21). These results highlight how crucial it is to preserve gut integrity to stop or minimize PD progression.

1.4. Alterations in the metabolites produced by the gut microbiota

Microbial metabolites are closely linked to the advancement of PD and may serve as markers of the gut microbiota's makeup and functioning. A connection between microglia cell activation and α-synuclein clinical symptoms is linked to aberrant microbial metabolites, and these factors can exacerbate the mobility abnormalities and neurodegeneration observed in PD rodent models (45).

In addition, SCFAs are the primary metabolite of gastrointestinal microflora's fermentation of dietary Fiber (which includes acetic acid, propionic acid, and butyric acid). This fermentation process is crucial for preserving the structural strength of the gastrointestinal epithelium, controlling immunological response, GI permeability, and brain function (46). The microbial metabolite concentrations among PD patients indicate the extent of pro-inflammatory gut microorganisms and the high number of proinflammatory microorganisms like *Ruminococcus sp*. and *Clostridiales bacterium* are significantly correlated with the rise in plasma SCFA levels, particularly propionic acid, and the fall in fecal SCFA levels (47). Furthermore, preclinical investigations on several animal models of PD have disclosed alterations in the gut flora and metabolites. Restoring a healthy gut microbiota in these animals has been shown to significantly lessen dopamine (DA) neuronal damage. Tyrosine hydroxylase (TH) and propionic acid expression were both downregulated in MPTP-induced mice models with lower *Faecalicatena* abundance (48). Propionic acid was able to trigger neuritic outgrowth in a PD model caused by rotenone. In addition to this impact on neurite outgrowth, propionic acid under rotenone treatment has the potential to markedly enhance the amount of TH-positive dopaminergic (49). According to another study, Sodium butyrate (NaB) improved gastrointestinal dysfunction and motor deficiencies in mice given rotenone. Sodium butyrate, on the other hand, shielded the colon and substantia nigra from rotenone-induced α-synuclein production and stopped the destruction of TH-positive neurons. In PD rats, NaB may also vary the constitution of the GM, govern the metabolism of gut SCFAs, and increase GLP-1 concentration in the serum, colon, along with substantia nigra (50).

1.5. Neurotransmitters and the microbiome

Gut bacteria can regulate biological activities and behavior in the animal hosts via physiological interactions via the nervous system, involving "direct" and "indirect" interactions. Apart from being able to produce some of the neuroactive substances themselves, microorganisms can also induce the host to produce additional metabolites and neurotransmitters that control gut-brain transmission. The brain's microglia need the microbiota to properly mature, activate, and develop. When bacterial-derived SCFAs are given to germ-free (GF) mice, microglia's morphology and function are restored, suggesting that signals from microbial metabolism oversee immunological training by microglia. Microbial-derived chemicals provide signals to the brain (51, 52). Microbial-derived chemicals provide signals to brain cells. The gut microbiota generates neurotransmitters like DA, norepinephrine, serotonin, glycine, and gamma-aminobutyric acid (GABA), each exerting distinct effects on brain GABA. Conditions may result from these neurotransmitter variations such as Parkinson's, Alzheimer's, autism spectrum disorder, anxiety disorders, and depression (53).

As an example, *Bifidobacterium infantis* affects central serotonin transmission by raising blood plasma tryptophan levels. *Lactobacillus* and *Bifidobacterium* are capable of producing GABA. *Bacillus, Saccharomyces*, and *Escherichia species* are able to produce noradrenaline. Serotonin production is attributed to *Streptococcus, Escherichia, Candida,* and *Enterococcus species*. Various bacteria can synthesize DA, and Lactobacillus can produce acetylcholine. Gut microorganisms produce SCFAs, 5-HT, DA, butyric acid, and gamma amino acids (54, 55). These neuroactive metabolites, which include neurotransmitters such as GABA, DA, NA, and 5-HT, along with amino acids like tryptophan and tyramine, LPS, long-chain fatty acids (LCFAs), SCFAs, trimethylamine-N-oxide (TMAO), and polysaccharide A (PSA), may directly or indirectly

cause peripheral immune cells to spread into the brain. This process is believed to result in neuroinflammation and impact central nervous system (CNS) functions (56, 57).

1.6. Prebiotics

Prebiotics are indigestible dietary elements that preferentially stimulate the development and/or activeness of good bacteria in the colon, having a positive effect on the host. They have demonstrated the potential to lower inflammation and alter the makeup of the gut flora (58). Prebiotics may be able to lessen systemic inflammation and enhance the function of the gut barrier, which could aid in mitigating a few of the peripheral and central symptoms of PD (59). Prebiotics might aid within the framework of rotenone-induced PD models by supporting a balanced gut microbiota, which may lessen systemic endotoxemia and gut permeability (60, 61). In doing so, it may be possible to prevent neuroinflammation and neurodegeneration by reducing TLR4-mediated inflammatory responses (62, 63).

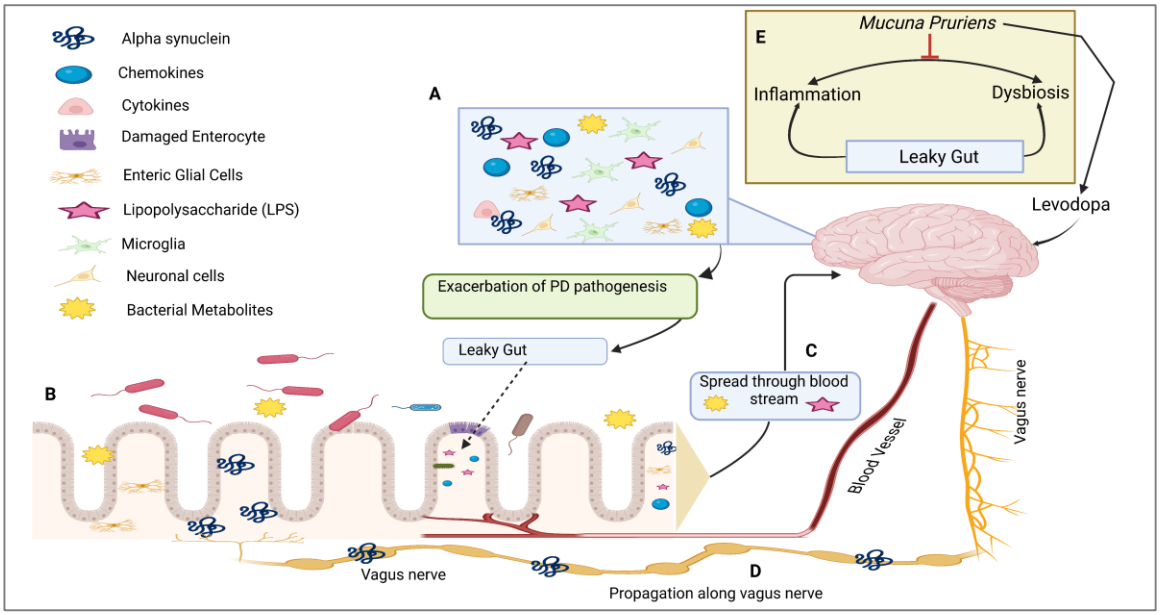
In summary, the etiology of rotenone-induced PD is significantly influenced by the colon, with pathways involving elevated intestinal permeability, gut dysbiosis, and systemic inflammation (10). It has demonstrated the promise for therapeutic therapies centered around GBA, as demonstrated by investigations on human and animal models (64, 65). New approaches like the use of prebiotics along with other drugs including Levodopa may become successful in managing PD as our understanding of these mechanisms increases, underscoring the significance of gut health maintenance in the setting of neurodegenerative diseases (66). Mucuna pruriens may function as prebiotics, as demonstrated by our earlier research. Furthermore, it has been established that levodopa, oligosaccharides, and other phenolic compounds are the elements of MP (28). We have thus concluded that it may have favorable therapeutic effects.

Hypothesis

Mucuna Pruriens shows the neuroprotective effect in Parkinson's disease by modulating gut dysbiosis in rotenone-induced Parkinson's model.

Rationale

Rotenone produces gut dysbiosis in Parkinson's disease through the disruption of the equilibrium and integrity of the gut microbiota, cellular oxidative stress, inflammatory processes, injury to the enteric nerves, and alpha-synuclein accumulation. Given the significant role of the gut-brain axis in PD pathophysiology, particularly in models involving the pesticide rotenone, it has been shown that the treatments that focus on the structure and functioning of the intestinal microbiome, like prebiotics (*Mucuna Pruriens*), could mitigate gut dysbiosis, neuroinflammation, and neurodegeneration. The graphical representation of the hypothesis is shown below.



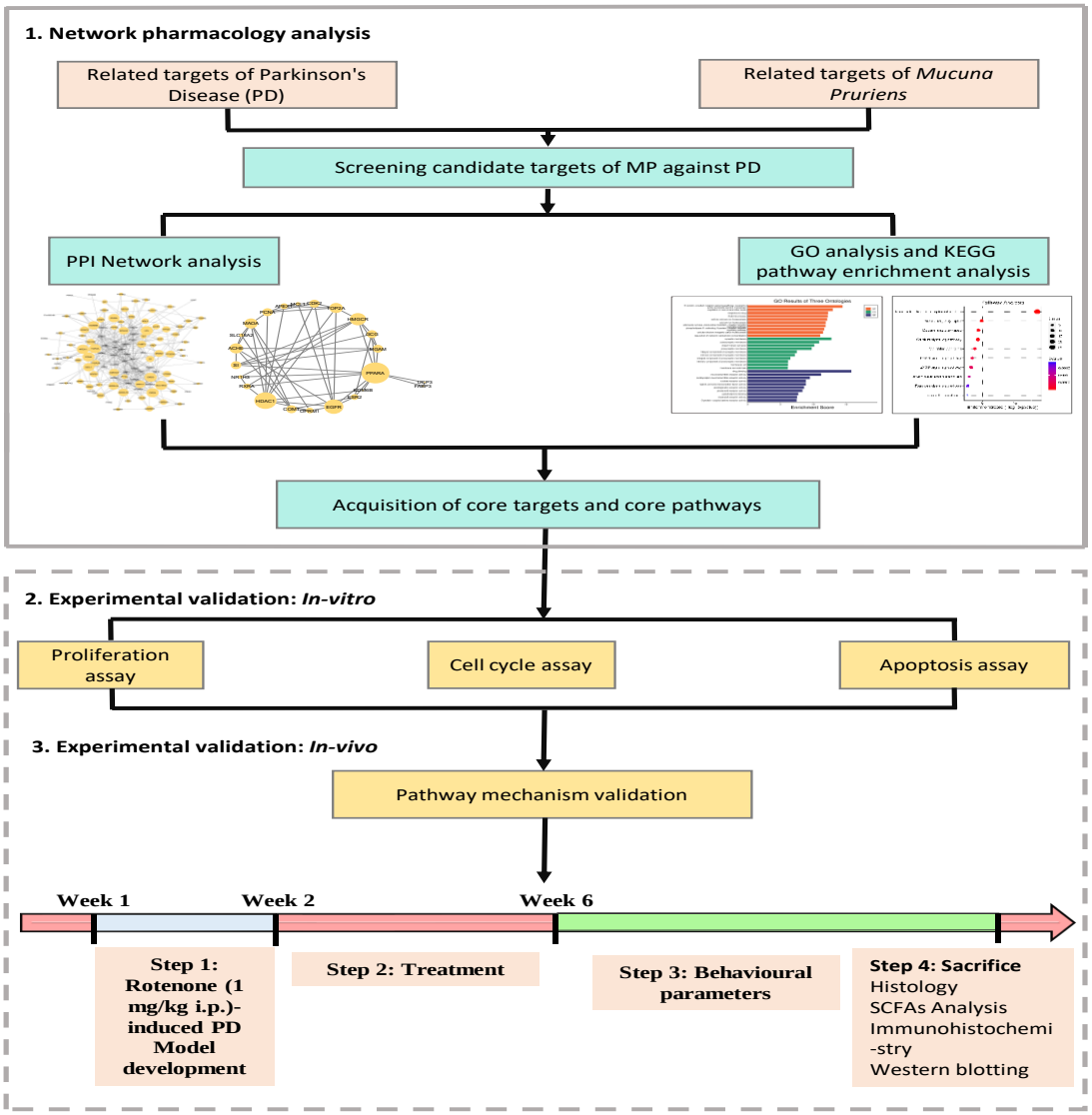
4. Aim and objectives

4.1. AIM: To assess the potential of *Mucuna pruriens* methanolic extract as a prebiotic in treating Parkinson's disease induced by rotenone.

4.2. OBJECTIVE:

- To find out the potential therapeutic targets of MPE and SCFAs in PD using the *in-silico* assays.
- To evaluate the *in-vitro* safety profile MPE on the SHSY5Y cell line.
- To evaluate the anti-parkinsonism effects of MPE in a rotenone-induced PD animal model.

PLAN OF WORK



METHODOLOGY

6.1. Materials

DMEM high glucose medium obtained from Invitrogen (Gibco # Cat.11965092), 1% antibiotics (Streptomycin + Penicillin; Invitrogen # Cat. 15140122), 0.05% Trypsin-EDTA (ThermosFisher # Cat. 1), Fetal bovine serum (FBS). SOD assay kit (# Cat. 19160-1kt-F) Pierce™ ECL Western Blotting Substrate (# Cat. 32209), PVDF Western Blotting Membranes (# Cat .3010040001), was purchased from Sigma-Aldrich Inc, USA, SHSY5Y cell line**,** HEK293 cell line, Methanol (Finar; # cat. 1093LC250, Phosphate-buffered saline (Sigma; #Cat. 806552), DMSO, MTT, TBS (Tris buffer saline) solution, ASO, Blocking reagent (Non-fatty acid (5%) in TBS), BSA standards. Antibodies namely GLP-1 (# Cat .1206R), and NFkB (# Cat .PA5-27617), were procured from Thermo Fischer and A SYNUCLEIN, (# Cat. PA1-18264) obtained INVITROGEN. OH-1, PARKIN 1. All the other chemicals utilized were of analytical grade.

6.2. ANIMALS

In this study, we obtained adult male C57BL/6 mice of ages between from the NIN, Hyderabad. Prior to the experiment, the mice were given a week to acclimatize to the environment. Throughout the experiment, the mice were provided with standard laboratory food and drinking water and were maintained at std. laboratory circumstances of temperature 23 ± 2° C with 12-hour day-night cycles. All animal tests were conducted in harmony with the guidelines set forth by CPCSEA, and with IAEC approval. IAEC protocol no. is **‘NIPER-H/IAEC/40/22’.**

6.3. NETWORK PHARMACOLOGY

6.3.1. Chemical Composition Database and Active Compound Screening

The IMPAAT database (IMPPAT | IMPPAT: Indian Medicinal Plants, Phytochemistry And Therapeutics (imsc.res.in) was used to screen active MP compounds. The active components of MP were screened using the pharmacokinetic (PK) indices of absorption, distribution, metabolism, and excretion (ADME), which included oral bioavailability (OB) >30% and drug similarity (DL) ≥0.02 (67). The OB value primarily indicates the degree to which a medication may get through several obstacles to enter the bloodstream and be absorbed by the body. The degree of compositional similarity between the target molecule and the established drug is mostly reflected by the DL value, which may be used to exclude substances that are inappropriate for the medication's chemical makeup (68, 69). Then, SwissTargetPrediction (SwissTargetPrediction), as well as STITCH (STITCH: chemical association networks (embl.de)) data sets, were utilized to retrieve the target genes associated with the drug's active components.

6.3.2. Acquisition of Critical Genes Associated with PD

Within the GEO database, the terms "Homo sapiens," "normal brain tissue," and "Parkinson's disease" were utilized as keywords. Functional groups with over-expression and under-expression (GSE8397, and GSE28894) were created by sorting out the genes that expressed differentially in Parkinsons brain tissues compared to normal brain tissues. To get the name and gene ID entering the UniProt database (https://www.uniprot.org/), the acquired gene list was imported.

6.3.3. In Silico Bioavailability, Drug-Likeness, and Toxicology Prediction

Using pk data SWISS ADME (SwissADME) and Molsoft (Molsoft L.L.C.: Drug-Likeness and molecular property prediction.), oral bioavailability and drug similarity were established at ≥30% and ≥0.02, respectively, to acquire appropriate herbal compounds. The PubChem database (PubChem (nih.gov)) was utilized to obtain the respective compounds' two-dimensional chemical

structures. The pharmacokinetics section provided a linear technique for determining the the permeability coefficient of skin (Kp). It is based on Potts and Guy's discovery that Kp is linearly associated with molecular size and lipophilicity (R 2 = 0.67). The greater the negative log Kp (measured in cm/s), the lower the skin permeative of the ligand (70). Pre-screening investigations for drug-likeness were conducted using Lipinski's "rule of five" (Pfizer) (71, 72), Ghose (Amgen) (73), Veber (GSK) (74), Egan (Pharmacia) (75), and Muegge (Bayer) (76). To ensure the medications are safe for human usage, we performed toxicity prediction on the selected 4 compounds. ProTox-II, a virtual lab for predicting small molecule toxicity, was used in the analysis (77). The medications were uploaded to the server and compared to previously reported pharmaceuticals like hydrochloroquine and remdesivir to forecast their toxicity.

6.3.4. Construction of Network

Cytoscape 3.10.2 (http://www.cytoscape.org/) is utilized to create "drug-disease" networks of CRC target genes by combining active components target genes, SCFAs genes, and disease-related genes. The drug compound-disease-target networks were constructed by utilizing the merge function analysis on core compounds. The network diagram depicts MP's active components or genes/proteins as nodes, with lines representing interactions among them.

6.3.5. Analysis of Protein-Protein Interaction (PPI) Network

At the highest medium level (0.4) for MP, SCFAs, and CRC-related targets, protein-protein (PPI) interaction networks were built using the STRING database platform, which also allowed target proteins to be rejected regardless of the network. All targets were restricted to the Homo sapiens species. Each solid circle in the PPI diagram represents a gene. Next, in order to determine the important genes, 3 topological characteristics" degree," "betweenness," and "closeness"—were computed. This option, "Degree," indicates how many edges are connected to a certain node.

"Betweenness" shows the quantity of shortest pathways. connecting two nodes, whereas "closeness" is the opposite of the number of distances (78, 79, 80).

6.3.6. Analysis of GO and KEGG pathway enrichment

One important bioinformatics tool for annotating genes and examining their biological functions is Gene Ontology (GO). The extensively used Kyoto Encyclopedia of Genes and Genomes (KEGG) database contains vast amounts of information about drugs, biological processes, diseases, chemicals, and genomes. Gene functions may be categorized into three types using GO analysis: cellular component (CC), molecular function (MF), and biological process (BP). This approach is frequently helpful for large-scale functional enrichment research (81). In this investigation, the biological roles of target proteins and pathways relevant to PD were derived by doing KEGG pathway evaluations and GO enrichment analysis. MP inhibits Parkinsonism by controlling the biological processes of target proteins or associated signaling pathways. To illustrate the data, the bubble graph and histogram are generated simultaneously.

6.4. MPE in Parkinson's disease: An experimental validation

6.4.1. Preparing MP Seed Extract (MPE)

As shown, the extraction of Mucuna pruriens was done according to an adapted method described in the literature with some modifications. The seeds were mechanically ground into a fine powder after being dried at room temperature [27˚C]. By using methanol: water (1:1 v/v) and shaking for 48 hours in a shaker incubator at room temperature, Mucuna pruriens hydroalcoholic extract was prepared. The residual material was removed by filtration and pooled followed by concentrated using Rota vapor. To get the MP powder extract, the samples were lyophilized for 24 hrs at 80˚C and 0.02 bar of pressure (82).

6.4.2. Assessment of DPPH Radical Rescue Operation

The MP's capacity to scavenge free radicals was assessed using the stable DPPH. After dissolving the dried MP sample in saline, it was filtered through a membrane with a pore size of 0.45 µm. Test tubes were filled with varying quantities of BHT, crude extract, and standard (+)-ascorbic acid (10 µg/ml). All of the tubes' volumes were brought to 100 µl by adding distilled water. After adding a 400 µl DPPH (100 µM) solution in ethanol to each tube, they were all violently shaken. For twenty minutes, the tubes were let to endure at room temperature. As previously mentioned (83, 84), a control was created without samples or standards. To adjust for the baseline, ethanol was utilized. At 517 nm, the absorbance variations for each of the tests and standards were recorded. There were three iterations of the experiment. A lower absorbance of the reaction mixture signifies a stronger capacity to scavenge free radicals. The inhibitory percentage, which was utilized to convey the radical scavenging activity, was calculated using a particular formula.

$$\textbf{Radical scavenging activity (\%)} = (OD_{control} - OD_{test})/OD_{control} \times 100.$$

6.4.3. Cell Culture

The neuroblastoma cells (SH-SY5Y) were cultivated at 37 ◦C in 5% CO2 using DMEM/F-12 supplemented with 10% heat-inactivated fetal bovine serum and 1% P/S (100 mg/mL streptomycin, 100 U/mL penicillin).

6.4.4. Cell Viability

The MTT test was used to identify cell viability. The test relies on living cells' capacity to change MTT into insoluble formazan, the quantity of which is correlated with the number of living cells. In T-25 culture flasks, the cell lines SH-SY5Y were initially cultivated. Once the cells reached 70% confluence, they were plated (1×10^4 cells/well) onto 96-well plates using 100 µl medium for each cell. Following a 24-hour incubation period at 37°C, cells were subjected to several

concentrations of MPE (0, 100, 200, 400, 800, and 1000 µg/ml) and left to incubate for 12, 24, and 48 hours. Each well was filled with 100 µl of 5 mg/ml MTT reagents after the medium had been fully withdrawn and cleaned with DPBS. After covering the plates with aluminium foil, they were incubated at 37 °C for four hours. Then the medium was thrown away. One hundred DMSO was used to dissolve the resultant formazan dye. At 570 nm, optical density (OD) was immediately measured using an ELISA reader. By using IC50, the impacts of MP on SH-SY5Y were ascertained.

To ascertain MPE's neuroprotective impact, SH-SY5Y cells were exposed to rotenone (10 µM), and rotenone (10 µM) in combination with MPE (100 µg/ml), and then incubated for 12, 24, and 48 hours. The MTT test was also used to evaluate the vitality of the cells (84).

6.4.5. Hoechst staining

Cells that have perished by apoptosis often exhibit compressed DNA and fractured nuclei, which may be distinguished from necrosis by staining the cells using Hoechst 33342 (Crowley, Marfell, & Waterhouse, 2016). In the present study, rotenone was administered to SH-SY5Y cells (5×10^5 cells/plate) with or without MPE. After being pretreated with 250 and 500 µM rotenone, the SH-SY5Y cells were subjected to MPE treatment. The Scale bar refers to 40 µM.

6.4.6. Experimental design

The first group was used for water vehicle control. For three weeks, rotenone dissolved in 2% CMC (I.P. 2 mg/kg) was administered into the second group. For four weeks, the third (LD) and fourth (HD) groups received oral dosages of MPE, namely 100 mg/kg and 200 mg/kg, respectively, while the fifth (STD) group received oral Semaglutide at a dose of 2.87 mg/kg body weight.

6.4.7. Neurobehavioral Assessment

6.4.7.1. Tail suspension test (TST) and forced swimming test (FST)

To evaluate the depressed behavior in mice, TST was used. wherein, in a dark room, animals are made to hang by their tails on a TST box hook with the use of tape. A stopwatch was used to measure out the immobility time of the mice throughout a 6-minute observation period, with the first three minutes being accounted for as agitation and the remaining three minutes as the immobility period.

FST is a commonly employed alternate technique for assessing depressive-like behavior in lab mice. Each mouse was housed separately in a long, cylindrical reservoir of water with a constant temperature of 27±1 ◦C. Using a stopwatch, the immobility duration of mice made to swim for three minutes was noted (85).

6.4.7.2. Open field test (OFT)

In an open-field test with animals, the behavioral effects of MPE on locomotor activity were assessed. The open field equipment is partitioned into nine square units, and all of the mice were positioned in the center. The locomotor activity of the mice was monitored for five minutes. The mice's total trip distance and total time spent immobile are measured in this 5-minute video. ANY-maze is used to record videotapes. U.S.A. behavioral tracking software version 5.0.

6.4.8. Histopathology

Following the sacrifice, a portion of the colon was taken out and preserved in a 10% formalin solution. Colon tissues were then subsequently eluted using gradient alcohols, submerged in xylene for a single hour, followed by paraffin-embedded. The colon samples fixed in paraffin were cut into 7 μm thin slices and stained with Hematoxylin and Eosin (86).

6.4.9. Western Blot Analysis

Brain tissue samples were collected, weighed (~100 mg), and added to the protease and phosphatase cocktail inhibitors containing RIPA lysis buffer until further use. The samples then

homogenised and to the supernatant, Laemmle buffer and β-mercaptoethanol were added kept for heating at 95 ◦C for 7 min. BCA method was employed for the protein estimation of the supernatant collected. When the samples get prepared, equal amounts of proteins were run were subjected to gel electrophoresis was carried out for the separation of the protein in 10% SDS polyacrylamide gel at approximately 40 µg. Further, Proteins were electro transferred onto to the PVDF membrane. These protein blots were cut according to the molecular weight concerning the protein ladder followed by blocking with 3% BSA in Tris buffer saline solution for 1 hr. After blocking, the blots were washed with Tris-buffered saline with Tween 20 (TBST, pH 7.4) for an hour at room temperature. The blots were then incubated with different primary antibodies mainly β-Actin, GLP-1R, NFKB, alpha-synuclein, and OH-1 at appropriate dilution for around 8-10 hr or overnight maintained at 40C. Then washed with TBST about 2–3 times for 15 min duration each and incubated in the dark at room temperature with HRP conjugated secondary antibodies. The β-Actin antibody is used as a loading control for normalization. Normalization of the band intensity of proteins was confirmed with the loading control protein. The β-Actin antibody is used as a loading control. ECL detection kit (Biorad, U.S.A.), was used to develop the blots and visualized the pictures to capture images by Fusion FX vilber lourmat ChemiDoc system. ImageJ software was used to analyze the band intensity.

CHAPTER 7

5. RESULTS

7.1 Network Pharmacological Analysis of MP Targeting CRC

7.1.1. Construction and Analysis of MP-SCFAs-CRC Targets Network

In this investigation, we extracted four compounds from MP with oral bioavailability (OB) ≥30% and drug-like (DL) ≥0.02 as evaluation criteria, utilizing the IMPAAT database (Table 1). Apart from that, we have also included four SCFAs such as formate, acetate, propionate, and butyrate. A total of 335 relevant genes were tested from these 8 compounds (from both MP and SCFAs). Three GEO (GSE8397, and GSE28894) datasets containing 5,211 genes linked to PD were searched (duplicates were deleted.) Using Venny 2.1.0, 120 common targets—genes from both MP and SCFAs—were acquired; these intersections were taken into consideration as possible candidate targets for MP and SCFAs against PD (Figure 1A). The Cytoscape 3.10.2 program includes 8 active ingredients (both MP and SCFAs) and 120 "drug-SCFAs-disease" critical target genes. An active ingredient-PD target network was created (Figure 1B). Several interactions are indicated by larger node sizes. Beta-sitosterol, and levodopa in regard to MP, Acetate, and propionate regarding SCFAs were the active components in MP and SCFAs, with the highest number of target genes respectively.

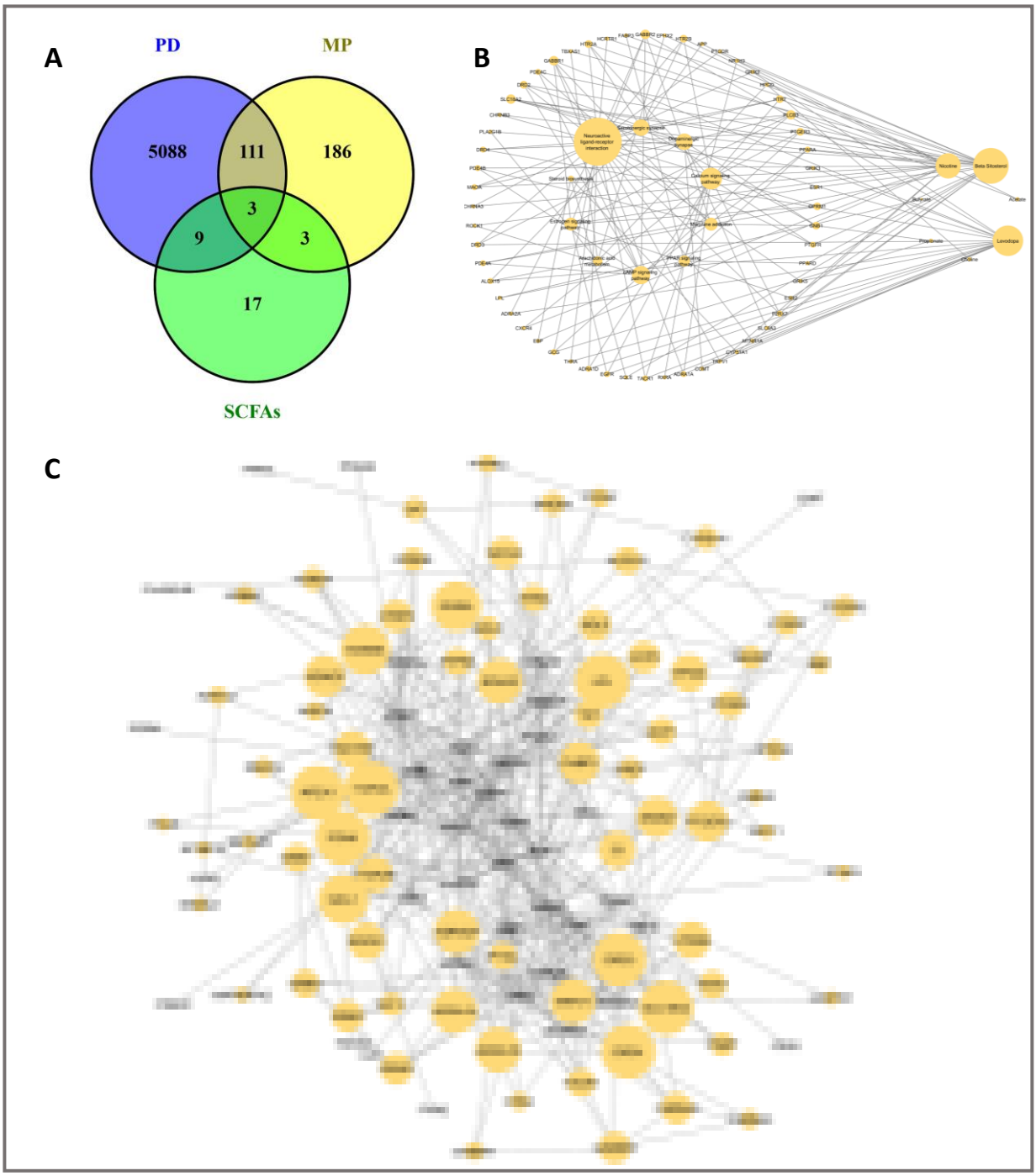


FIGURE 1 | (A) Venn diagram displays the 120 common targets of MP in Parkinson's disease (PD). (B) Compounds-SCFAs-disease-target network of MP against PD created by Cytoscape 3.10.2 software. (C) Cytoscape 3.10.2 program visualizes protein-protein interactions that were detected using STRING software.

7.1.2. In Silico Bioavailability, Drug-Likeness, and Toxicology Prediction

Drug-likeness (DL) investigations assess the likelihood that a molecule would operate like an oral medication regarding bioavailability (87). We carried out in-silico ADME investigations by utilizing the SwissADME online tool (87). The foresight of such features is critical in the drug development procedure, and inadequate ADMET attributes frequently fail around 60% of novel chemical entities during clinical investigations (88). As a result, SwissADME is an extremely useful and dependable tool. In silico approaches for predicting ADMET features are more appealing than traditional experimental assays due to the enormous number of substances (both real and developed) that can be investigated, as well as considerable time and cost savings. Compounds' oral bioavailability is demonstrated via bioavailability radar plots (Table 1).

The compounds nicotine and choline were predicted to be orally bioavailable. Beta-sitosterol and Levodopa displayed one offshoot relative to insolubility (INSOLU) and unsaturation (INSATU), respectively; this suggests that their oral bioavailability may be compromised by less-than-ideal physicochemical characteristics.

Table 1: Four main active ingredients of MP have drug-likeness (DL) and oral bioavailability (OB). Compounds 1–4 have been plotted using radar. The pink region has optimal physicochemical properties in regard to oral bioavailability. Lipophilicity (LIPO): -0.7 < XLOGP3 < 5.0; SIZE: 150 g/mol < MW < 500 g/mol; Polarity (POLAR): 20 Å2 < topological polar surface area (TPSA) < 130 Å2; and Insolubility (INSOLU): 0 < LogS < 6; INSATU (in-saturation): 0.25 < fraction of carbons in sp3 hybridization < 1; FLEX (flexibility): 0 < number of rotatable bonds < 1. The SwissADME web application was used to create the radar plots (87).

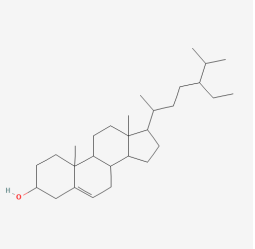
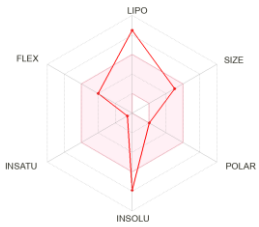
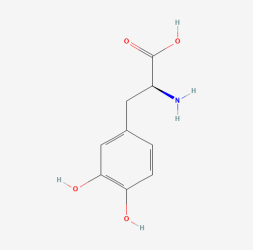
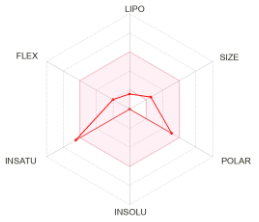
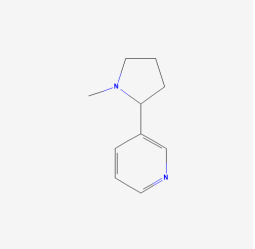
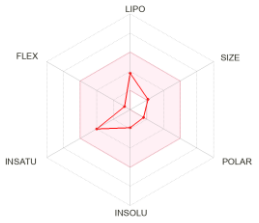
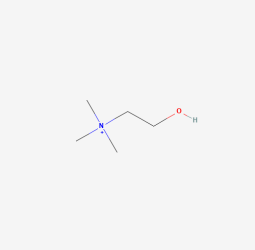
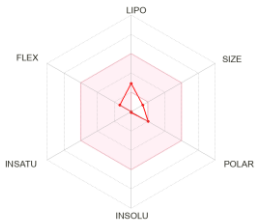
S.No.	Molecule Name	Structure	DL	OB (%)	Radar Plot
1	Beta-Sitosterol		0.29	0.55	
2	Levodopa		0.58	0.55	
3	Nicotine		0.03	0.55	
4	Choline		0.02	0.55	

Table 2 visually displays the GI absorption and blood-brain barrier (BBB) permeability in relation to absorption and distribution attributes, as well as other pharmacokinetic variables.

Table 2: The characteristics of substances' pharmacokinetic and medicinal chemistry

Pharmacokinetic Attributes	**Compounds**			
	Beta-Sitosterol	**Levodopa**	**Nicotine**	**Choline**
GI Absorption [a]	Low	High	High	Low
BBB [b] Permeation	0	0	1	0
PGP [c] Substrate	0	0	0	0
CYP [d] 1A2 Inhibitor	0	0	0	0
CYP [d] 2C19 Inhibitor	0	0	0	0
CYP [d] 2C9 Inhibitor	0	0	0	0
CYP [d] 2D6 Inhibitor	0	0	0	0
CYP [d] 3A4 Inhibitor	0	0	0	0
Log Kp (Skin Permeation) [e]	-2.2	-9.45	-6.46	-7.22

[a] Gastrointestinal; [b] Blood-brain barrier; [c] P-glycoprotein; [d] Cytochrome P450; [e] cm/s; 1 Yes; 0 No

The ligands were predicted to be toxic or not by using the online tool ProTox-II. ProTox-II (77) categorizes toxicity into six classes (1 to 6). Class 1 has a lethal LD50 of ≤5, while class 6 has an LD50 of >5000, indicating that the molecule is not poisonous. It also provides a prediction accuracy in terms of percentage. The study indicated that Beta-Sitosterol, Levodopa, and Nicotine were the best components among the nominated compounds. Table 3 displays the predicted medication toxicity parameters.

Table 3: Compounds that were shortlisted and whose toxicity assessment was completed with the ProTox-II tool.

Compounds	**Beta-Sitosterol**	**Levodopa**	**Nicotine**	**Choline**
Predicted LD50 (mg/kg)	890	1460	3	1391
Toxicity Class	4	4	1	4
Prediction Accuracy (%)	70.97	100	100	100
Hepatotoxicity [a]	0.87	0.58	0.95	0.94
Neurotoxicity [a]	0.54	0.73	0.67	0.55
Nephrotoxicity [a]	0.89	0.52	0.92	0.82
Respiratory Toxicity [a]	0.82	0.94	0.73	0.85
Cardiotoxicity [a]	0.85	0.8	0.93	0.69
Carcinogenicity [a]	0.60	0.66	0.91	0.28

[a] Probability

7.1.3. Target Protein-Protein Interaction (PPI) Network Analysis

The STRING database platform was utilized to create the target PPI network, which included 120 target genes from eight different active compounds. Homo sapiens was the selected species, and the PPI network had a medium 0.4 confidence level, indicating potential MP targets against PD. The string data was then loaded into the Cytoscape 3.10.2 program, and any free nodes were eliminated. Figure 1C depicts the PPI network, which is composed of 197 hub nodes and 359 edges (nodes removed which were free). The results reveal four cluster subnetworks based on MCODE assessment, with the cluster subnetwork with the greatest score consisting of 23 hub nodes and 57 edges. The clusters with the highest score of 5.182 comprised PPARA, HDAC1,

EGFR, HMGCR, ACHE, TOP2A, MAOA, SI, PCNA, GCG, COMT, CDK2, MGAM, OPRM1, APEX1, RXRA, ESR2, NR1H3, MCL1, SLC18A2, FABP3, KDM6B, UCP3 (Figure 2A). Figure 2B illustrates the identification of the most significant 10 core targets.

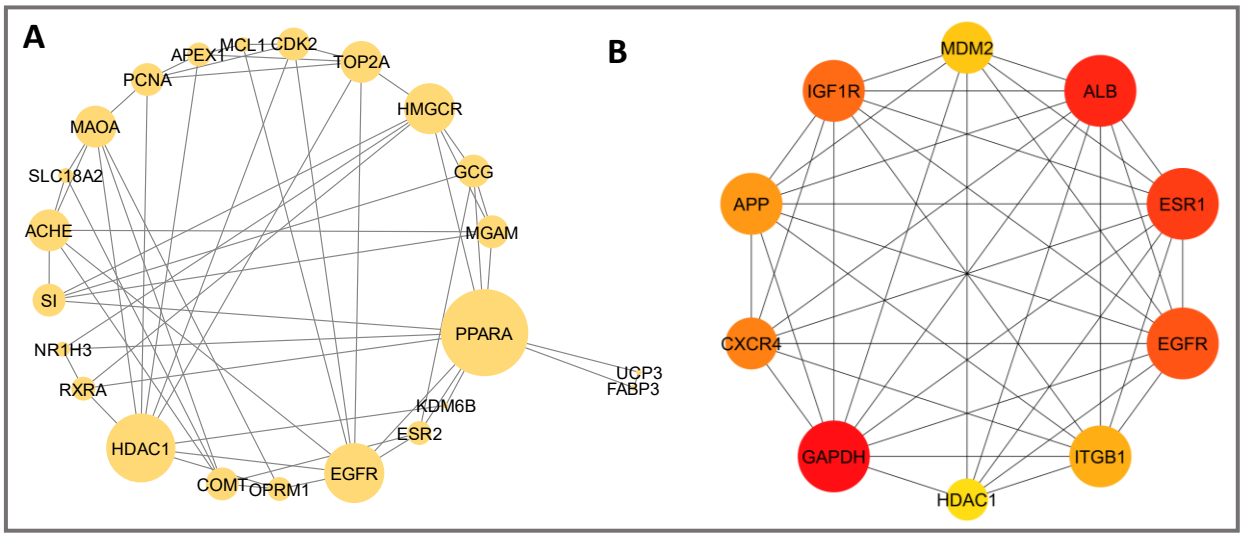


FIGURE 2 | (A) MCODE examination of a cluster subnetwork yielded a score of 5.182. (B) Top ten core nodes obtained. The size of the node, and the node's saturation, represent the interactions corresponding to each node.

Only the genes exhibiting greater "degree," "betweenness," and "closeness" values (above the median value) were gathered as the primary MP targets for PD based on the network analysis.

7.1.4. Predicting Functional Enrichment Analysis for MP

To look into the possible MP and SCFA targets for PD therapy mechanisms, The KEGG pathway and GO enrichment studies were carried out. The p-value < 0.05 was met by 1173 biological processes (BP), 85 cellular components (CC), 185 molecular functions (MF), and 46 KEGG pathways. Figures 3A, B, and C show the top 10 substantially enriched GO terms in BP, CC, and MF, respectively. The top 10 significantly enriched KEGG Pathways include Neuroactive ligand-receptor interaction, Serotonergic synapse, Dopaminergic synapse, Calcium signaling pathway,

Morphine addiction, PPAR signaling pathway, cAMP signaling pathway, Arachidonic acid metabolism, Estrogen signaling pathway, Steroid biosynthesis, and are shown in Figure 3D.

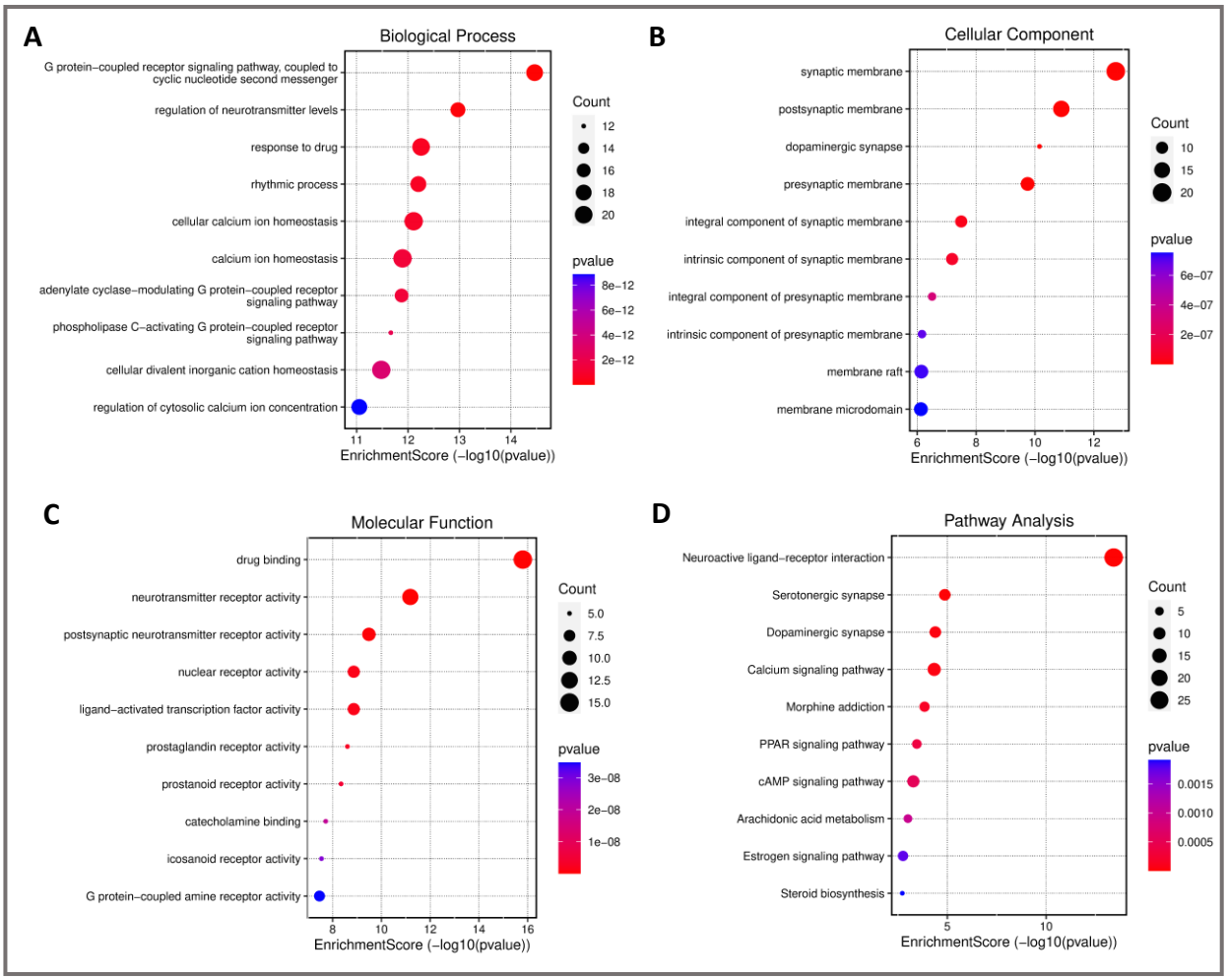


FIGURE 3 | Biofunctional enrichment study of Gene Ontology (GO) of Mucuna Pruriens (MP): A. Top 10 biological processes, B. Top 10 cellular components, C. Top 10 molecular functions in the GO analysis, (D) Top 10 KEGG pathways. The size of the dots denotes the counts of genes that connect to each term, and the color scale shows the various p-value criteria.

7.2. MPE in Parkinson's disease: An experimental validation

7.2.1. Phytochemical Potential and Antioxidant Capacity

The chemical compounds polyphenols and flavonoids, which are naturally occurring antioxidants with the ability to scavenge free radicals, found in the seed extract prevent oxidative stress-induced neurodegeneration. The extracts' potent ability to scavenge free radicals is demonstrated by the DPPH radical scavenging activity test. DPPH assay has been performed with different drug concentrations of 5,10,25,50 and 100 ug/ml with the standard ascorbic acid 10 ug/ml. The IC50 value was 44.20 ug/ml of MPE. The chemical compounds polyphenols and flavonoids, which are naturally occurring antioxidants with the ability to scavenge free radicals, found in the seed extract prevent oxidative stress-induced neurodegeneration.

7.2.2. Cell viability assays

Note: The graph and description part need to be added

7.2.3. Hoechst Staining shows the apoptotic stages

When cultivated cells are stained, the number of apoptotic cells rises in the rotenone group relative to the control. Furthermore, a greater number of Hoechst-positive apoptotic cells with fragmented and condensed nuclei in the DC group, while the lesser number of cells underwent apoptotic, has been observed in the treatment group with higher doses in a concentration-dependent manner (Figure 4). All values were expressed in Mean ± SEM. Statistical analyses were done using one-way ANOVA followed by Bonferroni's multiple comparison test. Analysis of the data was performed by GraphPad Prism 8 software. The level of significance was taken as $P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$.

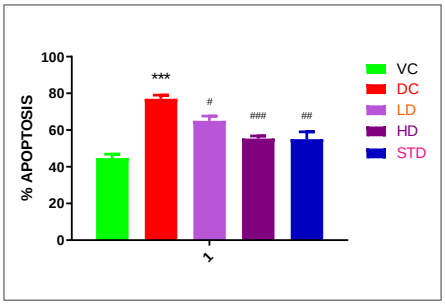


FIGURE 4 | Hoechst staining showing that high dose and standard drug lead to less percentage of apoptosis as compared to low dose and disease control.

7.2.4. MPE treatment restored the rotenone-induced anxiety and depressive kind of behavior in mice

To assess the impact of MPE treatment on rotenone-induced depression in mice on day 0 and day 28, On day 28 we measured their locomotor activity in OFT, TST, and FST. Rotenone significantly increased immobility duration in OFT (Fig. 5B), while MPE intervention significantly increased the frequency of central and peripheral crossings and rearings at lower dosages (100 mg/kg) and greater dosages 200 mg/kg ($P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$.). However, in the case of TST and FST, which demonstrate that it comprises depressive-like behavior, our results indicated that the rotenone-induced raised immobility period was considerably decreased upon treatment with MPE at both doses—100 mg/kg ($p < 0.05$) and 200 mg/kg ($p < 0.001$) (Fig. 5C and D).

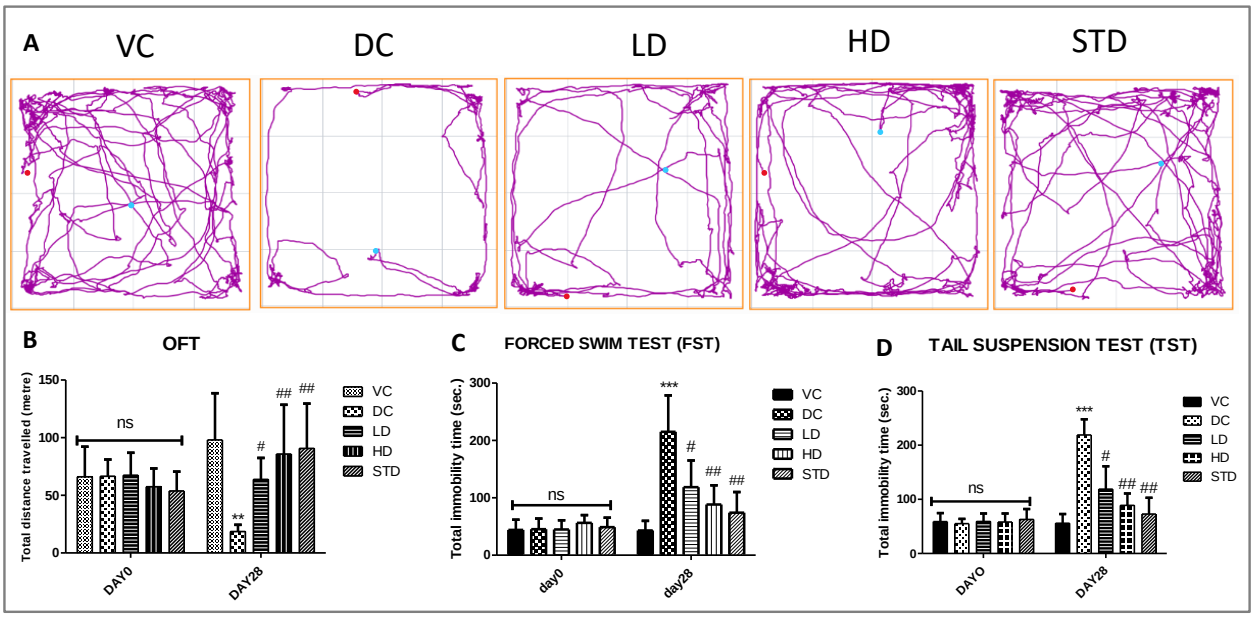


FIGURE 5 | MPE's impact on the behavior. A. On Day 28, typical head track plots from the Open Field Test (OFT), B. The total traveling distance in meters for OFT, C. Forced Swim Test (FST) (TST), D. Test of Tail Suspension

7.2.5. Histopathology

Hematoxylin and eosin staining show higher fold increase in (yellow color arrow) neutrophil infiltration, (red color arrow) goblet cell depletion, and (green color arrow) higher disruption of the intestinal mucosal lining. Low-dose groups have shown less improvement while the High-dose group has shown better results which is near to the standard group where the reduction of the inflammation, and improve the intestinal mucosal lining (Figure 6).

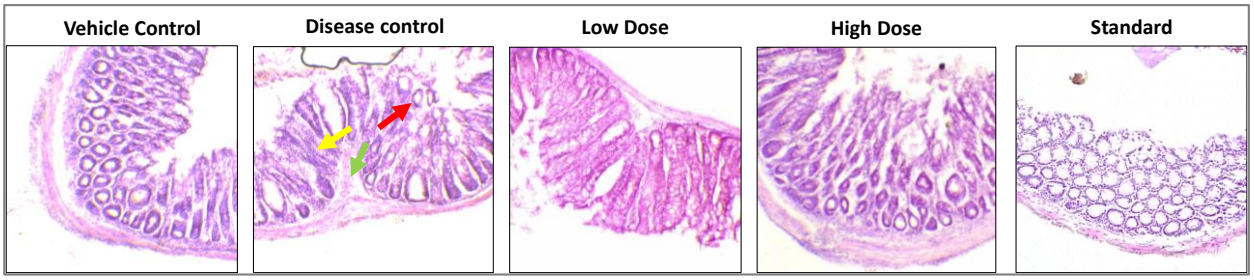


FIGURE 6 | Hematoxylin and eosin staining

7.2.6. Western blotting:

All values were expressed in Mean ± SEM. Statistical analyses were done using one-way ANOVA followed by Bonferroni's multiple comparison test. Analysis of the data was performed by GraphPad Prism 8 software. The level of significance was taken as *P < 0.05, **P < 0.01 and ***P < 0.001. We investigated the protein expression levels in mice brain tissue. The initiation of inflammatory signaling cascades heavily depends on activating the NF-κB transcription factor.As per our results accelerated level of it has been noticed in the DC group with the reversed effect has been noted in treatment group with higher dose significantly. An upregulation of GLPR expression at the MP-low dose. Furthermore, the higher MPE dose significantly increases the expression level of GLPR protein compared to the DC group. According to a previous study, there will be an overexpression of Parkin-1 protein. In our study, we observed a significant downregulation of protein expression in the treatment group compared to the disease control

group. MPE administration dramatically promotes anti-oxidant markers, including OH-1, in a dosage-dependent way. The process of neurodegeneration starts when the α-syn protein is overexpressed and clumps together. Conversely, the elevated levels of alpha-synuclein were significantly downregulated by MPE treatment at 100 mg/kg and 200 mg/kg in a dose-dependent manner (Figure 7).

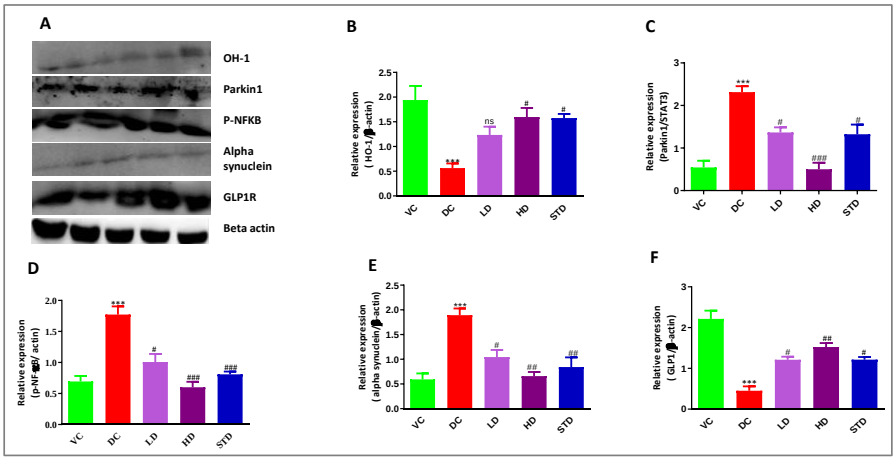


FIGURE 7 | A. Typical blots for **OH-1, Parkin-1, p-NF-κB, Alpha-synuclein, GLP-1, and β-actin B**. Quantification of OH-1 levels using western blots, represented graphically. C. Parkin-1 levels D. p–NF–κB levels E. Alpha-synuclein levels F. GLP-1 levels. Band intensities were evaluated using Image J software and normalized with β-actin. Data is provided as Mean ± SEM (n = 3). Statistical significance was evaluated using one-way ANOVA accompanied by the Bonferroni multiple comparison test.

DISCUSSION AND CONCLUSION

MP exhibits notable advantages in the clinical study and has high anti-PD potential in toxin-induced PD models. MP has strong anti-PD action because of its high L-DOPA content. Although several Parkinson's research has so far demonstrated that MPE has significant anti-parkinsonian effects, the possible mechanisms behind these benefits have not yet been thoroughly investigated (27). In order to understand the complex connections between herbs and illnesses at a systematic level and to adhere to a systematic and holistic approach, network pharmacology integrates bioinformatics, systems biology, and pharmacology. To investigate the possible bioactive

components of MP, we used two OB and DL. Studies on the pharmacokinetics of MP have been few thus far. It has shown that the constituents of MP (mainly Beta-sitosterol and, Levodopa) and, SCFAs (mainly acetate and propionate) target dopaminergic synapses which may be useful in the treatment of PD. We identified 4 active compounds from MPE and 120 possible targets in total for our study. We also showed that our herb is a synergistic one with multi-component, multi-target, and multi-pathway properties. The compound-target network revealed that the primary elements were SCFAs (propionate and acetate) and MP (beta-sitosterol, levodopa). In addition, the PPI network revealed data on co-expression and homology of proteins as well as information about the origin of the interactions. The biological network as a whole, comprising targets including PPARA, HDAC1, EGFR, HMGCR, ACHE, TOP2A, MAOA, SI, PCNA, GCG, COMT, CDK2, MGAM, OPRM1, APEX1, RXRA, ESR2, NR1H3, MCL1, SLC18A2, FABP3, KDM6B, and UCP3, was significantly impacted by MP, according to our PPI study. The findings of the enrichment also suggested that the mechanism by which MP prevents Parkinson's disease (PD) may be related to the coordinated regulation of multiple pathways, including the cAMP signaling pathway, the neuroactive ligand-receptor interaction, the calcium signaling pathway, the dopaminergic synapse, and other interconnected pathways.

We also investigated the neuroprotective effects of MPE on locomotor function, anxiety-like behavior, and antioxidant capacity in mice with ROT-induced PD at two doses: 100 mg/kg and 200 mg/kg. MPE's neuroprotective activity was also evaluated against ROT-induced neurotoxicity in SH-SY5Y cells. MPE exhibits antioxidant and neuroprotective properties in mice and SH-SY5Y cells, modulating the expression of alpha-SYN, OH-1, Parkin-1, p-NF-κB, and GLP-1 in PD illness. We looked at the effectiveness of MPE in the developed model of PD and discovered that it lowered the phenotypic and pathological features of the condition. MPE's anti-oxidative and

anti-aggregative properties support dopaminergic neurons' survival, proliferation, and differentiation.

As an addendum to previous research on medications against PD, this study used a network method to detail how MP chemicals modify several pathways against PD. Additionally, we showed that MP has a significant impact on several PD-related targets. We fervently hope that our study will contribute to the use of network pharmacology in elucidating the possible mechanisms of anti-parkinsonism herbs and offer insights into how herbs operate in concert to treat other complicated illnesses. From a critical perspective, this study does have certain shortcomings, though. Further tests (Western blot, immunofluorescence on cell lines analysis) are required to firmly confirm our findings, as this work was based only on data analysis. Furthermore, more research is required to confirm the possible benefits of MP for gut health and neuroprotection, even if this has only been mentioned in a small amount of literature. In the meanwhile, studies on the pharmacokinetics of MP against PD are required to demonstrate its properties.

Future Directions: As mentioned above there are some limitations in this study briefly network pharmacology have shown various enriched genes like PPARA, HDAC1, EGFR, HMGCR, ACHE, TOP2A, MAOA, SI, PCNA, GCG, COMT, CDK2, MGAM, OPRM1, APEX1, RXRA, ESR2, NR1H3, MCL1, SLC18A2, FABP3, KDM6B, UCP3, DRD3, and DRD4. These genes will have to be evaluated for a proper understanding of the mechanism behind MP in the treatment of PD.

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